Studies on *Avena sativa* L. seedlings, with particular reference to the role of indole-3-acetic acid contained in the endosperm

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ERRATA

p.10 para 3, L.9:	Zellstreckungs, not Zellstreckugs.
p.38 para 3, L.3:	Hewlett-Packard, not Hewitt-Packard.
p.41 para 2, L.3:	specific gravity, not activity.
p.46 para 2, L.3:	"in conjunction (with) ..."
p.64 para 3:	not IAA metabolites, but rather IAA breakdown products.
p.73 para 1, L.3:	from, not for
p.201 para 2, L.3:	treatment with, not of
p.210 para 1, last line:	"... is not unequivocal ...," rather than "... is not equivocal ..."
p.211 para 1, L.4:	"... of IAA or 'X' or 'Y' ...", rather than "... of IAA or 'Y' ..."

CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

ABA	Abscisic acid
Anova	Analysis of variance
Bq	Becquerel (1 nuclear disintergration per second)
conc	Concentrated
cv	Cultivar
GA_3	Gibberellic acid
GC-Ms	Combined gas chromatography - mass spectrometry
GLC	Gas-liquid chromatography
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IAAsp	Indole-3-acetylaspartic acid
IAM	Indole-3-acetamide
LSA	Liquid scintillation analysis
LSD	Least significant difference test
POPOP	1,4-bis[2-(5-phenyloxazolyl)]-Benzene
p.p.m.	Parts per million
PPO	2,5-diphenyloxazole
radio-GLC	Combined radioisotope counting and gas-liquid chromatography
s.e.	Standard error
TLC	Thin-layer chromatography
UV	Ultra Violet

Chromatography solvent abbreviations given in section 2.5.7.1

ABSTRACT

The role of free indole-3-acetic acid (IAA) in the endosperm of Avena sativa L. cv. 'Terra' seedlings, was investigated to determine its contribution to free IAA in the shoot.

IAA-2-^{14}C (in amounts equivalent to 177, 17.7, or 1.77 ng IAA), was injected into the endosperm of 63 h old etiolated seedlings, and transport and metabolism of the ^{14}C-labelled compounds determined over 24 h. It was concluded that translocation of free IAA directly from the endosperm, is probably not a significant source of free IAA in the shoot; mainly because even small amounts of IAA-^{14}C introduced into the endosperm, were rapidly metabolised. This suggested that in Avena, free IAA does not normally exist in the liquid endosperm. The two main hypotheses on the source of free IAA in cereal shoots (i.e. the grain, or synthesis in the coleoptile tip) are critically evaluated. The suggestion is made that both may serve as sources of IAA, with the relative importance of each possibly varying between species (since the auxin economies of Avena and Zea appear to differ). It is argued that IAA supplied from the grain may be derived from hydrolysis of high molecular weight IAA-esters at the endosperm-scutellar interface, rather than from direct transport of IAA-ester (cf Epstein, Cohen and Bandurski, 1980).

A major metabolite in the grain was a mild alkali-labile IAA - conjugate with some properties similar to the IAA-glucoprotein reported by Percival and Bandurski (1976).

Considerable destruction of IAA-^{14}C (25% in 15 min)

occurred on silica gel TLC plates, incubated in normal laboratory lighting prior to development. Breakdown was exacerbated by time and light.

The effects on seedling extension of endosperm injections of unlabelled IAA, gibberellic acid (GA_3), kinetin, or abscisic acid (ABA), with or without brief illumination, were determined; as were those of application to the coleoptile tip of IAA in agar. Histological studies were also conducted on treated seedlings.

1.0 INTRODUCTION

1.1 GENERAL

Seedlings of the Gramineae have been used in plant physiological studies for more than 100 years. Charles and Francis Darwin's (1880) early work on phototropism in canary grass (Phalaris canariensis) coleoptiles culminated in the demonstration by Went (1928), that an active substance could be isolated from Avena coleoptile tips. The substance, when reapplied to decapitated coleoptiles, promoted extension growth and was later shown to be indole-3-acetic acid (IAA). Despite years of study, the origin of the IAA in cereal seedlings, however, remains a controversial issue.

1.2 SEEDLING DEVELOPMENT

1.2.1 Structure of the Embryo

In the Gramineae, the seed coat or testa is fused to the pericarp and therefore the grain is technically a caryopsis. The embryo, which is embedded at one end of the grain, on the side away from the central furrow, is dominated by the scutellum - the structure which is responsible for supplying the developing embryo with nutrients from the endosperm. The embryonic axis consists of the coleorhiza, enclosing the primary root, and the coleoptile which encases the young leaves. Between these two structures lie initials for an elongation of the main axis, known as the mesocotyl or first internode.

The naming of this portion of the shoot is debatable, since many authors have attempted to describe the anatomy of the cereal seedling in terms of homologies with dicotyledonous seedlings. For example, if the scutellum is considered to be the modified first leaf of the plant and the coleoptile the second, then 'first internode' is appropriate for the interval between the two (e.g. Avery, 1930). However others (e.g. Went and Thimann, 1937) use the term mesocotyl in the sense of 'between cotyledons', assuming the scutellum is one

cotyledon and the coleoptile is the other.

According to Avery (1930) the term 'mesocotyl' as originally applied by Celakovsky, did not mean 'between cotyledons', but was used because he considered the structure to be neither hypocotyl nor epicotyl, hence 'mesocotyl'. Brown (1960) observed that in 160 years of study the mesocotyl has been variously interpreted as a 'neck', an elongated node, a rhizome, a fusion of the hypocotyl and part of the cotyledon, as well as many even more obscure structures.

Because of its widespread use in the literature, the term mesocotyl will be used in this thesis, without implying support for any particular dogma.

1.2.2 Elongation of the Shoot in Darkness

When oat (Avena sativa L.) grains are germinated in darkness both the coleoptile and mesocotyl elongate slowly for about 2d. From the third to the sixth day the mesocotyl extends rapidly and then its growth virtually ceases. Rapid elongation of the coleoptile is normally delayed slightly so that the 'grand' period of growth occurs approximately one day after that of the mesocotyl. By the 8th day after planting, extension of the coleoptile has usually also ceased.

The final length of the mesocotyl and coleoptile are usually approximately equal, although this length, and the relative growth rates of the coleoptile and mesocotyl, may vary between varieties (cf. Schneider, 1941 and Mer, 1951). The primary leaves contained within the coleoptile, continue to grow after coleoptile extension ceases, and break through the tip of the coleoptile.

Root initials are present in the early stages of development, at the coleoptilar node, and are carried up as the mesocotyl elongates. These initials grow out diageotropically to form crown roots which serve to anchor the young plant just below the soil surface (Goodwin, 1941).

1.2.2.1 Mesocotyl

Extension growth of the mesocotyl is accomplished by means of an intercalary meristem situated just below the coleoptilar node (Boyd and Avery, 1936). Seedlings of the Gramineae have been divided into 3 basic types according to the extent of elongation exhibited by the mesocotyl in darkness and the position of the intercalary meristem (Tateoka, 1967).

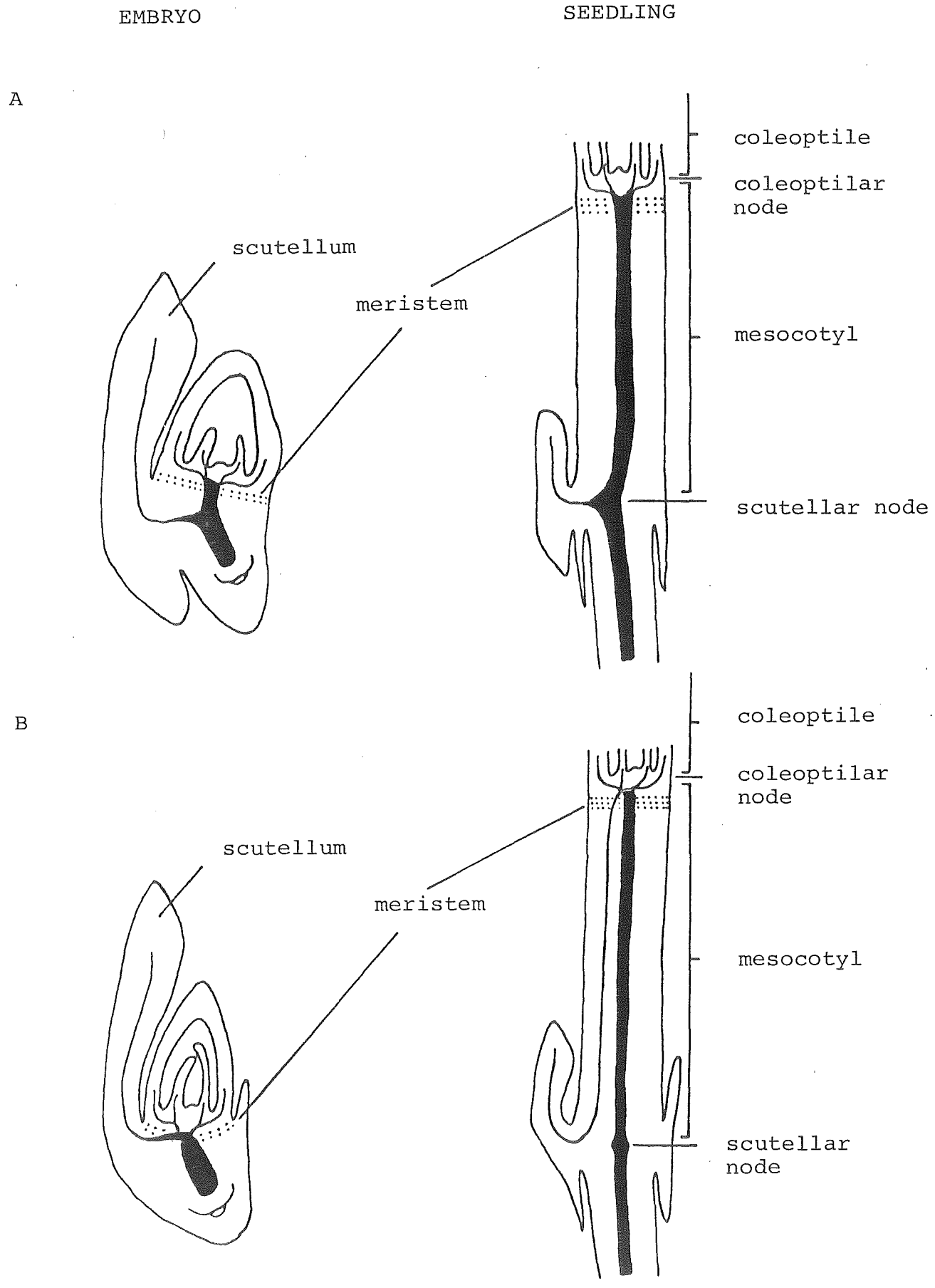
(a) Zea type. In this group the meristematic region in the embryo is located just below the coleoptilar node, but some distance from the scutellum. Consequently the scutellar trace is not altered as the mesocotyl develops, but runs directly to the central vascular stele. The result is a mesocotyl consisting of an epidermis, cortex and central stele (Fig. 1.1a).

(b) Avena type. The Avena-type seedling has a meristem which traverses the loop in the scutellar trace, so that as new mesocotyl tissues are formed this trace is perpetuated to form a cortical vascular bundle, parallel to the central stele, throughout the length of the mesocotyl (Fig. 1.1b). Thus in Avena the mesocotyl is comprised of a central stele surrounded by an epidermis and a cortex containing a single vascular strand. This runs from the scutellum up to the coleoptilar node where part of it turns down into the central stele towards the roots. The remainder diverges to form part of the two vascular strands on either side of the coleoptile. In the Avena-type seedling, no direct vascular connection is apparent between the scutellum and the roots, other than via the mesocotyl (Boyd and Avery 1936).

(c) Triticum - type. The position of the intercalary meristem is similar to in the Avena seedling. However virtually no elongation takes place, so that a mesocotyl is not readily discernible.

Some transitional forms are found between the Triticum- and Avena-types (Sargant and Arber, 1915; Hoshikawa, 1969).

Figure 1.1: Schematic drawings of longitudinal sections of the embryo and a portion of the seedling of (A) Zea-type and (B) Avena-type seedlings (After Tateoka, 1967)


EMBRYO
SEEDLING
A
scutellum
meristem
coleoptile
coleoptilar node
mesocotyl
scutellar node
B
scutellum
meristem
coleoptile
coleoptilar node
mesocotyl
scutellar node

1.2.2.2 Coleoptile

The coleoptile is essentially a hollow cylinder, 5-6 cells thick, with a solid conical top terminated by a small pore (Went and Thimann, 1937). Elongation is almost entirely in the longitudinal axis i.e. growth is polarised (Avery and Burkholder, 1936). In transverse section the coleoptile is elliptical, with the short axis in the plane of symmetry of the grain. The two small vascular bundles run up either side and terminate 0.5 - 0.7 mm from the coleoptile tip.

Cell division in the coleoptile is not concentrated in any particular region, but mitoses occur throughout the length of the structure, except at the extreme tip (0.5 mm). These cells neither elongate nor divide (Avery and Burkholder, 1936). It must be stressed that the coleoptile tip is not meristematic and it should not be confused with an apical meristem (as was done by Trewavas, 1981).

There is some doubt regarding the duration of cell division in the dark-grown coleoptile. Thomson (1954) found that division stopped on the fourth day after planting. Mer and Causton (1967) obtained conflicting results. In one case cell division followed a pattern similar to that described by Thomson; in other cases, mitoses persisted for at least 7d.

1.2.3 Effect of Light on Development of the Shoot

Exposure to light results in inhibition of mesocotyl extension, acceleration of coleoptile development, and the earlier appearance of crown roots. According to Goodwin (1941), light inhibition of mesocotyl growth was first reported by Rothert in 1896. Three responses have been distinguished in this inhibition (Blaauw, Blaauw-Jansen and van Leeuwen, 1968).

(a) The first response, which reduces mesocotyl elongation by up to 15%, is extremely light sensitive. The degree of inhibition is proportional to the logarithm of the light dosage given, and the response is saturated by 10^{-1} $\mu W\ sec.cm^{-2}$ at λ = 660 nm. The inhibition is not reversed

by far-red irradiation and all wavelengths of light in the visible spectrum, including green light, have been shown to cause inhibition, although red light is the most effective. Hence green 'safelights' are not morphogenetically-inactive (Schneider, 1941; Goodwin, 1941; Weintraub and McAlister, 1942; Huisinga, 1964; Mer, 1966; Mandoli and Briggs, 1978/79).

(b) The second response of mesocotyl suppression, which reduces elongation to between 85 and 50% of the dark control, is phytochrome mediated and far-red reversible. Inhibition is linear for red light quantities between 10 and 10^4 $\mu W\ sec.cm^{-2}$, after which the response becomes saturated.

(c) The third response inhibits mesocotyl elongation to approximately 5% of the control. It is dependent on the duration of irradiation rather than the total quantity of energy and is not reversed by far-red illumination.

Both the first response (Mer, 1966) and the second response (Goodwin, 1941) are associated with the suppression of cell division. Cell elongation is inhibited by longer exposures to light (Goodwin, 1941).

The three inhibitory steps in the elongation of the mesocotyl are matched by three increases in the growth rate of the coleoptile, although the final length is reduced (Blaauw et al., 1968).

Araki and Hamada (1937), and Goodwin (1941) have shown that the light stimulus which affects mesocotyl inhibition, can be recepted at any part of the shoot; although illumination close to the coleoptilar node was found the most effective (Araki and Hamada, 1937).

1.3 DEVELOPMENT OF THE HORMONE CONCEPT IN COLEOPTILES

The concepts of plant physiology as we know them today are generally considered to have their origins in the ideas advanced by Charles Darwin in his book, 'The Power of Movement in Plants', published in 1880. Darwin and his son Francis

had experimented with seedlings of canary grass. They observed that coleoptiles bent towards unilateral light. If, however, the plants were decapitated, or the tips shaded, no such bending occurred. As a result of their studies the Darwins concluded that "when seedlings are freely exposed to a lateral light some influence is transmitted from the upper to the lower part causing the latter to bend".

The subsequent work that led to the development of the hormone concept postulated by Went in his thesis in 1927, is well known and is covered by numerous reviews (e.g. Boysen Jensen, 1936; Went and Thimann, 1937; Audus, 1972; Letham et al., 1978) the main findings are summarised below.

Fitting (1907) showed that lateral incisions, either on one or both sides of the Avena coleoptile, did not prevent the coleoptile curving towards unilaterally-applied light. Boysen Jensen excised the coleoptile tip, then replaced it after inserting a gelatin block between the tip and the stump. When the tip was illuminated unilaterally, the lower part of the coleoptile bent towards the light in the usual manner, indicating that the stimulus from the tip was transmissible through the gelatin. Furthermore, insertion of mica plates on the shaded side of the coleoptile prevented curvature, whereas normal curvature resulted when the mica was inserted on the illuminated side. This established that an influence on the dark side of the coleoptile promoted curvature towards light (cited Boysen Jensen, 1936; Went and Thimann, 1937).

Paál (1919) demonstrated that when an excised coleoptile tip is placed on one side of the Avena coleoptile stump, accelerated growth beneath the tip results in curvature, even in darkness. He proposed that the stimulus was a growth substance secreted by the tip, and that in darkness it diffused symmetrically down the coleoptile, but was distributed asymmetrically when the plant was illuminated unilaterally.

The concept that normal coleoptile growth was regulated by a symmetrically distributed growth substance, diffusing

from the tip, was confirmed by Söding (1925). He found that decapitation inhibited the straight growth of Avena coleoptiles, but that extension could be largely restored by replacement of the excised tip.

Went (1928) adapted a method originally devised by Stark, where plant extracts were applied to coleoptiles in small agar blocks. Went found that while a plain agar block would not restore growth when applied to the stump of a decapitated coleoptile, growth was renewed by the addition of agar which contained extracts from excised tips. Went then showed that the active substance could be collected from excised tips by diffusion into agar. The unilateral placement of the agar on a decapitated Avena coleoptile stump resulted in curvature which was proportional, within limits, to the number of tips or the time they were on the agar block. Went not only demonstrated the existence of a growth substance secreted by the coleoptile tip, but also developed a quantitative bioassay to measure it.

Went considered that the straight growth of the Avena coleoptile is controlled by two factors. One, the growth substance formed in the coleoptile tip (later termed auxin by Kögl from the Greek auxein, to increase), is transported basipetally and is used up while producing growth, so that an auxin deficiency in the basal regions causes their growth to decrease and eventually stop. The decline in the growth rate in the regions near the tip was explained by the shortage of another factor, termed Zellstreckugs material or food factor, which interacts with auxin to produce growth and which comes from the grain or the roots (see Went, 1935).

It should be noted that most of the studies leading to the development of this concept involved the growth of coleoptiles that had been either grown in diffuse light, or exposed to light to study phototropism, or to facilitate manipulations such as decapitation. As has been noted above (section 1.2.3), exposure to light causes inhibition of mesocotyl extension and the standard 'phototropically inactive' red/orange safelight used in early experiments emitted light

at a wavelength that is the most effective inhibitor of mesocotyl elongation.

In many experiments it was common practice to expose young Avena seedlings to red light to deliberately inhibit mesocotyl development, since this long, curved structure was considered a nuisance (e.g. Went and Thimann, 1937). Therefore most of the evidence cited above relates to the growth of plants in which the mesocotyl had been partially or totally suppressed. Furthermore exposure to light causes a promotion in the growth rate of the coleoptile (Blaauw et al., 1968).

1.4 IDENTIFICATION OF THE ACTIVE SUBSTANCE

1.4.1 Auxin a, Auxin b and Hetero-auxin

After Went had demonstrated the presence of auxin in Avena coleoptile tips, renewed efforts were made to isolate and identify the active substance. Because of the low amounts in plants, other auxin-rich sources were used to obtain sufficient substance for chemical identification.

Kögl and co-workers isolated and identified the crystals of three substances which had exceptional activity in the Avena curvature test (see Audus, 1972; Bearder, 1980). Auxin a (auxentrolic acid) and hetero-auxin (indole-3-acetic acid) were purified from human urine. Another substance, given the name auxin b (auxenolonic acid) was identified in malt and maize germ oil.

According to the formulae proposed, auxin a and b were previously unknown to chemical science. Indole-3-acetic acid (IAA) was long known to organic chemists and had been synthesised in 1905 by Ellinger. Thimann (1935) was able to show that the active substance ('rhizopin'), first isolated by Nielsen in 1930 from the fungus Rhizopus suinus, was in fact hetero-auxin (IAA).

Because the small amounts of growth substances in plants made direct chemical analysis difficult, indirect methods of identification were used. These mainly involved the

acid-alkali stability of the compound and diffusion constants in agar. The molecular weight of the naturally occurring active substance in the oat coleoptile, determined by the diffusion method, was found to be 378. This approximated with the theoretical molecular weight of pure auxin a(mol wt 328). On this basis, and with regard to the acid-alkali stability of the compound, the active substance in Avena was identified as auxin a (Went and Thimann, 1937).

1.4.2 Establishment of IAA as a Natural Auxin

IAA was finally isolated from a plant source in 1942 by Haagen-Smit, Leech & Bergren, who obtained it from an alkaline hydrolysate of cornmeal. This was corroborated by Berger and Avery (1944), who found IAA in maize kernels, as did Haagen-Smit and co-workers (1946). IAA has since been shown to be widespread, although not necessarily universal, in the plant kingdom but the number of unambiguous identifications remains small (for reviews of the occurrence of auxins see Audus, 1972; Bearder 1980).

Numerous attempts have been made to repeat the earlier isolations of auxin a and b, from both urine and plant sources, but without success. Despite this the concept of auxin a and b persisted until into the 1950's (e.g. Haagen-Smit, 1951), when the reliability of the identification methods came under suspicion (Audus, 1972).

Wildman and Bonner (1948) showed that when auxin obtained from Avena coleoptile tips by diffusion into agar was extracted from the agar into ether, a different diffusion constant was obtained which was very close to that of IAA. These workers then went on to show that the bulk of the native auxin in the coleoptile tip was IAA, and not auxin a.

More recently, a synthetic compound with the proposed formula of auxin b has been shown to possess little biological activity (Nakamura et al., 1966; Hwang and Matsui, 1968) and rigorous physical analysis (X-ray and mass spectroscopy) of the original samples of auxin a and b, preserved at the

University of Utrecht, has shown them to be cholic acid and thiosemicarbazide (Vliegenthart and Vliegenthart, 1966). Neither of these compounds has any auxin activity and the mystery of the growth-promoting activity of the fresh samples remains (Audus, 1972).

In Avena, IAA has been shown to be present - conclusively (by GC-MS), in extracts of seedlings (Bandurski and Schulze, 1974), and grains (Percival and Bandurski, 1976) - and by semi-specific methods, in coleoptile tip diffusate (Shen-Miller and Gordon, 1966), and guttation fluid (Sheldrake and Northcote, 1968; Sheldrake 1973).

IAA has been unambiguously identified in Zea coleoptile tip diffusate (Greenwood et al., 1972), seedlings (Bandurski and Schulze, 1974), roots (Elliot and Greenwood, 1974), and grains (Bandurski and Schulze, 1977).

1.5 CONTROL OF THE INTEGRATED GROWTH OF THE MESOCOTYL AND COLEOPTILE

As noted previously, Went's two factor theory for control of coleoptile extension was mostly developed using plants which had been exposed to light. The integrated growth of the coleoptile and mesocotyl, in plants grown in darkness, received less attention. Inevitably, elongation of the mesocotyl was also explained in terms of auxin produced in the coleoptile tip. This school of thought, promulgated by van Overbeek (1936), and Inge and Loomis (1937), was supported by the following evidence: treatments which inhibited mesocotyl extension also caused a decrease in the supply of auxin e.g. decapitation of the coleoptile (van Overbeek, 1936; Inge and Loomis, 1937), exposure to light (van Overbeek, 1936; Burkholder and Johnston, 1937) and heat treatment (van Overbeek, 1936; Inge and Loomis, 1937); while it was shown (in some cases, at least) that mesocotyl inhibition could be reduced or eliminated by application of synthetic IAA to the coleoptile tip or stump (van Overbeek, 1936; Inge and Loomis, 1937).

Kondo, Fujii and Yamaki (1969), who used a weak green 'safelight' to facilitate manipulations, found removal of the whole coleoptile from 65h old etiolated Avena seedlings resulted in inhibition of mesocotyl extension and that application of IAA to the stump restored the mesocotyl growth rate, in the following 12h period, to the same level as the control. However IAA application would not restart extension growth if the stumps were briefly irradiated with red light.

These workers also found that illumination of whole plants resulted in a large, but temporary, decrease in the amount of auxin secreted from the bases of coleoptiles excised above the node (and measured by the standard agar diffusion/Avena curvature test). If secretion was determined below the node then, in the illuminated plants, the auxin level remained low.

Studies using isolated shoot segments, which included the coleoptilar node, indicated that red light inhibited the transport of exogenously applied IAA-2-^{14}C through the nodal region, due mainly to immobilisation of IAA within the tissue. Kondo et al. (1969) concluded this decrease in transport across the node, rather than a decline in auxin production by the coleoptile, probably resulted in inhibition of mesocotyl elongation.

Schneider (1941), using a diffusion method similar to van Overbeek, found auxin production in light and dark-grown oat plants was identical, when measured by the Avena curvature test. Production in both increased to a peak 90h after planting, then declined; although the rate of decrease was more rapid in light-grown tips than in dark. Schneider concluded "auxin production of coleoptile tips of light- and dark-grown Avena seedlings correlates better with coleoptile growth than with mesocotyl growth". Also he was unable to markedly offset red light inhibition of mesocotyl extension by application of IAA in lanolin paste to the coleoptile tip. Application of IAA to the stump, after excision of the coleoptile just above the node, similarly caused only a small reversal of mesocotyl inhibition, even though it was obvious from the appearance of the tissue that large amounts of auxin had reached the mesocotyl.

Schneider considered it unlikely that light-inhibition of the mesocotyl was caused by effects on auxin production, inactivation or transport.

Mer (1951) was careful to grow his Avena seedlings in total darkness, performing his experimental manipulations without the aid of a 'safelight'. He found that while repeated removal of the coleoptile tip would decrease the growth of the coleoptile, mesocotyl extension was not significantly affected. In later experiments where the whole coleoptile, with or without the leaves, was excised from the partially swollen embryo, elongation of the mesocotyl was actually promoted (Mer, 1972).

In addition, Dattaray and Mer (1964) found the amount of ether-extractable auxin in the Avena mesocotyl, determined by the coleoptile straight growth bioassay, was not reduced by treatments such as red light exposure or heating (40°C, 3h), which inhibited mesocotyl elongation. In fact, high auxin contents were found to be present even when mesocotyl growth had ceased.

Araki and Hamada (1937) and Goodwin (1941) have established that irradiation of the coleoptile tip is not necessary to bring about mesocotyl inhibition; in fact localised illumination near the coleoptilar node is more effective than illumination of the tip (Araki and Hamada, 1937). Mer (1969) believes the coleoptilar node is the controlling centre of light inhibition of the mesocotyl and that illumination of other parts of the shoot may indirectly stimulate the node. He has shown that the etiolated oat seedling behaves like a glass rod to light impingeing upon it, so that the light is transmitted within the seedling from the point of entry.

By irradiating isolated Avena mesocotyl segments, it has been shown that the inhibition can be mediated directly by the mesocotyl tissue itself, and treatment with IAA will only partially reverse this effect (Schneider, 1941; Goodwin, 1941; Sandmeier and Nitsch, 1963). Sandmeier and Nitsch however claim the effect of red light is dependent on the

origin of the mesocotyl segments; that extension of nodal segments, and the 2 mm zone below the node is promoted by a brief exposure to red light, whereas segments taken from between 2 and 10 mm below the node are inhibited.

1.6 THE ROLE OF THE GRAIN IN THE AUXIN ECONOMY OF THE SHOOT

In most textbooks of plant physiology, the coleoptile tip is described as the site of auxin production where IAA is synthesised from tryptophan (or tryptamine) (e.g. Leopold, 1964; Wareing and Phillips, 1970; Black and Edelman, 1970; Audus, 1972). This view is based primarily on the work of Wildmann and Bonner (1948), who showed that Avena coleoptiles contain an enzyme system for the conversion of tryptophan to IAA and that the activity of this system was greatest at the coleoptile tip. That IAA is synthesised in situ at the coleoptile tip, has generally become accepted as the 'Classical' auxin hypothesis, synonymous with F.W. Went. However Went himself, like many of the earlier workers, recognised that the cereal grain played an important role in the formation of auxin in the Avena coleoptile. This was put clearly by Skoog (1937, p313) "It has long been assumed ... that the auxin synthesized in the tip of the coleoptile is derived from a precursor transported from the seed".

Pohl, who showed that the grain was rich in auxin, considered that the auxin in the coleoptile tip was transported directly from the grain (see Went and Thimann, 1937; Sheldrake, 1973). A similar view was promulgated by Avery and Burkholder (1936). Skoog (1937) attempted to intercept this auxin in Avena by removing the coleoptile tip and placing a block of agar on the stump. No auxin was detectable when the block was assayed by the deseeded Avena curvature test. If, however, the block was left on the test plant for 2 to 6 h then a distinct auxin curvature resulted; whereas no such curvature was apparent from plain agar blocks. This was interpreted as evidence that a precursor, present in the

block, was being converted into auxin, either in the agar block or at the cut surface. Skoog claimed that in other (unpublished) experiments he had excluded the possibility of activation through bacterial action.

Coleoptiles of deseeded plants were found to be more sensitive to unilaterally applied IAA, which was attributed partly to a decline in endogenous auxin production and partly to the maintenance of cell wall plasticity following the removal of most of the materials for secondary wall formation (Skoog, 1937).

Auxin production in the coleoptile tip was found to decline steadily following removal of the grain (Went and Thimann, 1937), and as further evidence that deseeding removed the materials for auxin synthesis, Skoog (1937) claimed to have shown that regeneration of the new physiological tip in decapitated coleoptiles was prevented.

It has been pointed out by Mer (1951), that Skoog's own data (Skoog, 1937, Fig 4A, p 318) show that deseeding does not prevent regeneration of the physiological tip (indicated by gradual straightening of the coleoptile in the Avena curvature test, Skoog, 1937), but merely delays its inception until more than 7h after decapitation compared to 2-3h in normal plants.

Furthermore, Sheldrake (1973) has shown that Skoog's failure to detect auxin in agar blocks placed on the apex of decapitated coleoptiles cannot be regarded as evidence against the acropetal movement of auxin from the grain to the coleoptile tip. Sheldrake found that when IAA-1-^{14}C was introduced into the transpiration stream of Avena coleoptiles, very little could be detected in agar blocks placed on the stump of the decapitated coleoptile, even though considerable amounts had accumulated at the new tip.

In contrast, Whitehouse and Zalik (1967) were able to detect IAA-1-^{14}C in agar blocks placed on decapitated Zea mesocotyls, after microinjection of labelled IAA into the endosperm of 3d old etiolated seedlings. However they gave no indication of what proportion of the applied IAA was transported.

Sheldrake (1973) has analysed the guttation fluid collected from the coleoptile of etiolated oat seedlings. He found two zones of auxin activity on chromatograms of the acid-ether soluble fraction of this fluid. One was identical to IAA; the other, which had a higher Rf in isopropanol/ammonia/water, was probably a precursor of IAA and could be converted by mild heating, to a compound with a mobility similar to IAA.

These compounds were present in guttation fluid from primary leaves, and also in guttation fluid collected from coleoptiles following repeated decapitation, indicating that auxin wasn't merely eluted from the tip (Sheldrake, 1973). Zea guttation fluid contained less of the high Rf compound but considerable quantities of alkali-labile complexes of auxin, probably esters, in addition to 'free' IAA.

Dye introduced into the transpiration stream appeared in the guttation fluid and also accumulated at the coleoptile tip. Similarly radioactive IAA injected into the endosperm of Avena grains was recoverable from the coleoptile tip and also was detected in the guttation fluid. This and other evidence led Sheldrake to conclude that auxin and auxin precursors move acropetally in the xylem, from the grain to the coleoptile tip, where the conversion of 'inactive' auxins to IAA takes place.

A compound similar to Sheldrake's 'auxin precursor' was obtained from aqueous diffusates of excised Avena coleoptile tips by Shen-Miller and Gordon (1966). They found IAA and two areas of low auxin activity, which they termed 'P' and 'F', in chromatographs of the acidic ether-extractable fraction of the diffusates. Both 'P' and 'F' were active in the Avena straight growth test, but not in the curvature bioassay. 'P' could be converted to an IAA-like compound in vitro by mild heating and had a UV spectrum similar to an indolyl complex. In light, the ratio of IAA to 'P' and 'F' was found to rise, due to a decrease in the amounts of 'P' and 'F' and probably an increase in IAA. Shen-Miller and Gordon concluded that 'P' and 'F'

were probably precursors of IAA, and the evidence indicated that the intact seedling was necessary for the maintenance of a 'P'-'F' pool at the tip.

Cereal grains were known to be rich in auxin, but the auxin yield could be increased manyfold by mild alkaline hydrolysis of either the tissue or the plant extracts. Berger and Avery (1944) showed that 95% of the potential auxin activity in the maize grain was in the form of an alkali-labile 'precursor' which was insoluble in water, ether, acetone and absolute alcohol but soluble in aqueous acetone, aqueous ethanol and aqueous alkali. Haagen-Smit *et al* (1942) established that the auxin released from the 'precursor' by the hydrolysis was IAA (the first isolation and identification of IAA from a plant source).

Alkali-labile 'precursors' of auxin were shown to be present in kernels of wheat (Avery, Berger and Shalucha, 1942; Haagen-Smit *et al*., 1942), rye (Hatcher, 1943) and oats(Haagen-Smit *et al*., 1942).

1.7 THE 'SEED AUXIN PRECURSOR' IN ZEA

Over the last 20 years, the nature and role of the 'auxin precursors' in *Zea* grains has been intensively studied by Bandurski and co-workers. These researchers have shown that the alkali-labile auxins in *Zea* kernels are esters of IAA, confirming a speculation made earlier by Went and Thimann (1937).

More then 16 different conjugates of IAA have been identified in *Zea* using modern methods of analysis such as combined gas chromatography - mass spectrometry (GC-MS). These conjugates include low molecular weight isomeric esters of IAA and *myo*-inositol, IAA-esters of *myo*-inositol glycosides, and IAA-esters of high molecular weight glucans (Bandurski, 1980).

Zea kernels contain high concentrations of auxin, between 72.0 and 79.5 mg total IAA per kg of tissue, of which about 10% is 'free' (i.e. acid-ether extractable),

and 90% is esterified (i.e. extractable after 1N alkaline hydrolysis)(Bandurski and Schulze, 1977). However these levels may vary between harvests (cf. Ueda and Bandurski, 1969), and between workers (cf. Epstein, Cohen and Bandurski, 1980). The IAA esters rapidly disappear from the grain during germination while free IAA levels remain relatively constant (Ueda and Bandurski, 1969; Epstein et al, 1980).

Through a series of isotope dilution experiments Bandurski and co-workers have calculated both the amounts and the rates of turnover of the indolyl compounds in the developing Zea seedling. In two recent publications they have presented a body of evidence which they claim shows that IAA-esters, rather than tryptophan or tryptamine, are the immediate precursors of IAA in the endosperm and shoot, and that the myo-inositol ester is the major transport form of IAA from endosperm to shoot (Nowacki and Bandurski, 1980; Epstein et al., 1980).

Radioactively-labelled IAA-myo-inositol was found to be transported from the grain into the shoot at 400 times the rate of labelled IAA and at 40 times the rate of IAA derived from labelled tryptophan; the latter two sources of IAA were shown to be insufficient to provide the needs of the developing shoot (Nowacki and Bandurski, 1980). Furthermore, application of labelled IAA-myo-inositol to the endosperm resulted in the appearance of 93% IAA-^{14}C ester and 7% free IAA-^{14}C in the shoot (Nowacki and Bandurski, 1980), and these are the normal endogenous proportions in Zea shoots (Bandurski and Schulze, 1977). A small amount of labelled IAA ester was also found to occur in the shoot after labelled IAA was applied to the endosperm of Zea seedlings (Hall and Bandurski, 1978). Bandurski's group therefore believe that the concentration of free IAA in the Zea seedling is controlled by the relative rates of synthesis and hydrolysis of the IAA esters (Bandurski, Schulze and Cohen, 1977; Bandurski 1980; Epstein et al., 1980; Nowacki and Bandurski, 1980).

The enzymes to make and hydrolyse the conjugates have been shown to exist in Zea (Hamilton, Bandurski and

Grigsby, 1961; Kopcewicz, Ehmann and Bandurski, 1974). *In vitro* IAA-*myo*-inositol synthesis (Kopcewicz *et al*, 1974) and hydrolysis (Hall and Bandurski, 1981) has been demonstrated using enzymes systems derived from *Zea*.

According to Bandurski (1980) the interconversion of free and bound IAA, or 'hormone homeostasis', may also be the mechanism by which environmental stimuli affect seedling growth. Indeed, Bandurski *et al* (1977) found that a brief flash of light, which inhibited *Zea* shoot elongation by 34%, resulted in a 42% reduction in free IAA, which was more than compensated for by a rise in ester IAA levels. However a similar experiment, looking at the effect of geostimulation of a maize shoot, was less definitive. Free IAA levels were found to be significantly higher on the lower side of the shoot, but a large standard error for the ester determination made it impossible to say whether the 'homeostatic' system was operating (Bandurski, 1980).

1.8 A 'SEED AUXIN PRECURSOR' IN *AVENA*?

The precursors of auxin in *Avena* have received less attention, and whether a system similar to that hypothesised for *Zea* also operates in this plant has not been established.

Percival and Bandurski (1976) have calculated that dehusked *Avena* grains contain a total of 8 $mg.kg^{-1}$ IAA and that about 95% of this is esterified (i.e. released by 1N alkaline hydrolysis at Room Temp. for 1h). The remainder of the IAA is in the free form, although a very small amount of IAA bound through a peptidic linkage is also present (released by 7N alkali, 3h, 100°C). The bulk of the ester lAA was shown to consist of high molecular weight compounds containing IAA, glucose, phenolics, and peptide, which Percival and Bandurski have designated IAA-glucoprotein.

In contrast, *Avena* seedlings contain mostly peptidyl IAA (75.5%), very little esterified IAA (5.5%), and 18% in the free form (Bandurski and Schulze, 1977).

Zimmermann, Siegert and Karl (1976) also determined free and bound IAA levels in Avena. In this case free IAA was obtained by Soxhlet extraction in methanol for 6h. Bound IAA was defined as that released by subsequent treatment of the plant tissue with 1N NaOH, for 48h at R.T. (for grains, at least, this probably gave a similar estimate to Bandurski and co-workers - see Ueda and Bandurski (1969) Fig. 1).

According to Zimmermann et al. grains with the husk intact contain 17.7 $mg.kg^{-1}$ total IAA, 93% in the bound form and 7% free. Total IAA was found to remain constant during germination and growth of the seedlings; there was simply a transfer from grain to seedling. After 4d 5.2% of the IAA was extractable from the seedling; 8.9% as free IAA and 91.1% as bound.

Part of the bound IAA was found to be water-soluble. In the grain this water-soluble bound IAA was associated with a protein with a molecular weight of 36,000 daltons, whereas the water-soluble fraction of the bound IAA in the shoot was a compound with a low molecular weight.

Zimmermann and co-workers concluded that no biosynthesis of IAA took place in etiolated seedling and that the bound IAA of the grain was transformed into the bound IAA of the shoot, perhaps with free IAA as an intermediate. The newly formed bound IAA was then probably transported to the coleoptile tip, where the physiologically active free IAA was liberated.

Bandurski and Schulze (1977) have measured free and bound IAA (ester and peptidyl) levels in a number of other plant species. It is notable that cereal grains contain high concentrations of IAA, particularly esterified IAA whereas legume seeds, contain less, mainly peptidically bound. Only in rice (Oryza sativa) has the nature of the esters been investigated further, and 10% of the total ester fraction was shown by GC-MS analysis to consist of IAA myo-inositol (Hall, 1980).

1.9 RATIONALE FOR, AND AIM OF, THIS INVESTIGATION

Dark-grown seedlings obtain all their organic requirements from the grain, and it is likely that the grain is also the source of IAA for the shoot - either directly or in the form of an IAA precursor.

Cereal grains are particularly rich in IAA with between 5 and 30% 'free' and the remainder esterified (Bandurski and Schulze, 1977). Very few studies have been carried out on the role of the IAA in the endosperm, especially the role of free IAA.

In Zea, Hall and Bandurski (1978) found that very little free IAA was detectable in the shoot 8 h after application of labelled IAA to the endosperm. Only 1.9% of the applied radioactivity was transported into the shoot, and of this only 0.6% was present as free IAA. An equivalent amount of esterified IAA was also present. However over 98% of the radioactivity in the shoot remained unaccounted for, and Hall and Bandurski made no attempt to identify it, other than to say it was neither free nor esterified. These workers were unable to conclude whether free IAA itself was transported into the shoot or whether IAA was released from a transport form once in the shoot.

Whitehouse and Zalik (1967) provided evidence that free IAA was transported acropetally into the shoot following microinjection of IAA-^{14}C into the endosperm of Zea, as labelled IAA was detectable in agar blocks placed on the stump of decapitated plants. However, these workers gave no indication of what proportion of the applied radioactivity was translocated, nor did they determine bound IAA-^{14}C levels.

In Avena, Sheldrake (1973) found IAA-^{14}C was translocated acropetally from the grain, and was detectable at the coleoptile tip and in the guttation fluid. Like Whitehouse and Zalik, Sheldrake did not quantify this transport, nor did he determine whether bound IAA forms were also present, but his studies of the endogenous auxins in the coleoptile tip and guttation fluid led him to believe that endogenous IAA 'precursors' were also transported acropetally from the endosperm.

Many workers (e.g. Went and Thimann, 1937; Skoog, 1937; Sheldrake, 1973; Zimmerman et al., 1976; Nowacki and Bandurski, 1980) have suggested that IAA 'precursors' move to the coleoptile tip where

free IAA is released. Sheldrake claims that labelled IAA, applied to the endosperm of Avena seedlings, also accumulates at the coleoptile tip, but careful analysis of his experiments shows that this may not be the case. Sheldrake did show that IAA-^{14}C was present at the coleoptile tip following injection into the endosperm; whether there was more or less at the tip relative to the rest of the coleoptile or shoot was not ascertained. What he did show was that IAA-^{14}C, fed via the roots or via the cut base of the coleoptile would build up at the coleoptile tip. However, the mesocotyl contains two vascular systems - the central stele (continuous with the root vascular system) and the cortical vascular strand, which comes from the scutellum. Hence IAA-^{14}C applied to the roots and to the endosperm travels in two completely different systems, at least as far as the coleoptilar node, where the two merge into one, and the twin coleoptile traces arise. Given the distinctly different functions of the two vascular systems, one supplying water and inorganic nutrients from the roots, the other supplying organic and nutritional requirements to the shoot from the endosperm, there is no reason to believe that the passage of root and endosperm applied IAA-^{14}C would be similar. Therefore Sheldrake has not shown that IAA from the grain accumulates at the coleoptile tip. Nor has it been shown that IAA 'precursors' from the grain accumulate at the tip.

In none of the studies where labelled IAA has been applied to the endosperm, has the subsequent distribution of label throughout the shoot been determined, nor have all the different forms of radioactivity in the tissue been accounted for. Therefore no comprehensive picture has been obtained of where the IAA-^{14}C from the grain goes or in what form it exists.

This project was concerned with investigating the role of IAA in the endosperm of Avena sativa during growth of the etiolated seedling. The transport and metabolism of radioactively-labelled IAA injected into the endosperm, was followed in detail, and particular emphasis was given to

identifying areas where radioactivity accumulated in the shoot, and to determining the rate of IAA metabolism in both the grain and the shoot. The effect on seedling development of applications of exogenous IAA to the endosperm was studied. In addition the response of seedlings to similar applications of other growth regulators was determined. The influence of light, which inhibits mesocotyl elongation, was also investigated with respect to its effect on transport and metabolism of labelled IAA, and with respect to the ability of the various growth regulators to reverse this inhibition.

2.0 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Avena sativa L. cv 'Terra', a 'naked' oat, was used in all experiments. Grain was obtained from Dr McKenzie, Agriculture Research Station, 195 Dafoe Road, Winnepeg, Manitoba R37 2M9, Canada, and from Crop Research Division, Department of Scientific and Industrial Research, Lincoln, New Zealand.

Grains were sterilised in 1.5% aqueous sodium hypochlorite (v/v)('Janola') for 5 min, rinsed in distilled water and soaked in 0.01 N HCl for a further 10 min to remove traces of adsorbed sodium hypochlorite (Abdul-Baki, 1974). The grains were then thoroughly washed in distilled water (normally 6-8 washes).

Seedlings were grown using a method similar to that devised by Mer (1953). Sterile grains were inserted, embryo-downwards, between heat-sterilised, folded blotting paper strips within 7 x 60 mm lengths of glass tubing. Tubes (3) were placed vertically in small vials containing approximately 6 ml of distilled water. Capillary action of the blotting paper supplied moisture to the germinating grain.

The tubes and vials were placed in specially modified metal containers and incubated in a darkroom at 24 $\pm$ 1°C. The containers (10 l lever-top cans, Alex Harvey Industries, Christchurch, New Zealand) were fitted with two ventilators which permitted air to circulate, but which excluded light. Humidity of 80 - 90% was maintained by wet paper towels placed on the floor of the metal cans. Normally approximately 90 seedlings were grown in each container.

2.2 LIGHTING

2.2.1 'Safelights'

The darkroom was equipped with two dim green 'safe-lights', which were used to aid certain experimental manipulations of the seedlings e.g. selection of seedlings, application of growth regulators. One large light was suspended approx 0.75 m above the work bench and consisted

of a 1.2 m long, green 40 W fluorescent tube, filtered through 8 layers of No. 39 Primary Green 'Cinemoid'. A smaller 30 x 25 cm box-'safelight' was used on the bench in an upright position, so that the lighted side could be used as a work surface. Light was provided by a 40 W incandescent bulb (Philips) filtered through one layer No. 1 Yellow, one layer No. 20 Deep Blue (primary), and two layers of No. 39 Primary Green 'Cinemoid'. The light intensity at the work surface was 0.06 nE.cm.$^{-2}$.s^{-1} (12.8 μW.cm^{-2}) (measured with a LiCor Model LI 185A photometer).

2.2.2 Red Light

In some experiments, seedlings were exposed to red light for 10 s. The light source consisted of two 1.2 m long, 40 W standard daylight fluorescent tubes, filtered through a single layer of No. 14 Ruby 'Cinemoid'. The intensity of the light was 0.65 nE.cm^{-2}.s^{-1} (141 μW.cm^{-2}).

2.3 APPLICATION OF GROWTH REGULATORS

Seedlings were selected 63 h after planting, for uniformity of shoot length. This was done by comparing shoot lengths with reference points marked on one of the safelights, and selecting those plants with shoots 30 - 35 mm long (approximately 1 seedling in 6).

Application of the growth regulators was either in agar blocks placed on the intact coleoptile tip, or by injection of solution into the endosperm. For the latter, 4 μl of test solution was injected into the centre of the endosperm, from the ventral side of the grain, using a 25 μl syringe (Unimetrics). A delay of about 4 s was allowed for dispersion of the injected fluid before the needle was withdrawn. Selection and injection of 25 plants took approximately 60 min.

The injection technique was used for experiments with radioactively-labelled IAA, as well as unlabelled IAA, gibberellic acid (GA_3), abscisic acid (ABA) and kinetin. Only unlabelled IAA was used with applications in agar.

2.4 PREPARATION OF GROWTH REGULATOR SOLUTIONS

2.4.1 IAA-2-^{14}C

Aliquots of IAA-2-^{14}C in toluene/acetone (1.998 GBq. mmol^{-1}, The Radiochemical Centre, Amersham) were dried under a stream of nitrogen in dim light and taken up in sufficient 10% aqueous ethanol (v/v) to give the required concentration of IAA-^{14}C. Solutions were prepared no more than 48 h prior to use, and were stored in darkness at 4°C.

2.4.2 Unlabelled growth regulators

Growth regulators were made up at the highest test concentration (2.5 x 10^{-3} M) and other solutions prepared by dilution.

GA_3, ABA or IAA (Sigma) were dissolved in 0.5 ml ethanol, which was then dispersed slowly in 80 ml of vigorously-stirred distilled water at 40°C. After 30 min stirring, the solutions were transferred to 100 ml volumetric flasks and made up to 100 ml with distilled water. Kinetin (Sigma) was dissolved in 0.5 ml of 0.1 N NaOH and dispersed in distilled water as above. The solution was neutralised with 0.1N HCl and made up to 100 ml. Control solutions of 0.5% aqueous ethanol (v/v), or 0.5 ml 0.1 N NaOH and 0.1 N HCl in water were also prepared.

All solutions were stored in darkness at 4°C. The GA_3, ABA and kinetin solutions were used within 6 weeks of preparation. IAA was made up fortnightly.

2.4.3 IAA in agar

IAA solutions were prepared in 1% agar (w/v) (Difco Bacto-) and after autoclaving (120°C for 15 min), 30 ml of molten solution were poured into 9 cm plastic petri dishes. Halves of gelatin capsules (4 mm diameter, Lilly and Co) were used to bore small cylinders of agar from the hardened plate. The agar was then pushed to the end of the half-capsule with a glass rod.

This method yielded cylinders 4.7 mm long and 4 mm in diameter (i.e. with a volume of approximately 60 mm^3). At an IAA concentration of 2.5 x 10^{-3} M, each cylinder contained 26.4 µg of IAA.

2.5 IAA-^{14}C TRACER STUDIES

The transport and metabolism of IAA-2-^{14}C was studied following injection into the endosperm of 63 h old dark-grown seedlings. In 3 series of time-course experiments, 4 µl injections were made containing:

(a) 2 kBq (177 ng) IAA-^{14}C
(b) 200 Bq (17.7 ng) IAA-^{14}C
or (c) 20 Bq (1.77 ng) IAA-^{14}C (see sections 2.3 and 2.4)

Injections were made under the 'safelights', and afterwards the plants were incubated for 1, 3, 6 or 24 h. Each harvest time within each series (i.e. dilution) was a separate experiment.

2.5.1 Injection of 2 kBq and 200 Bq IAA-^{14}C

2.5.1.1 Incubation

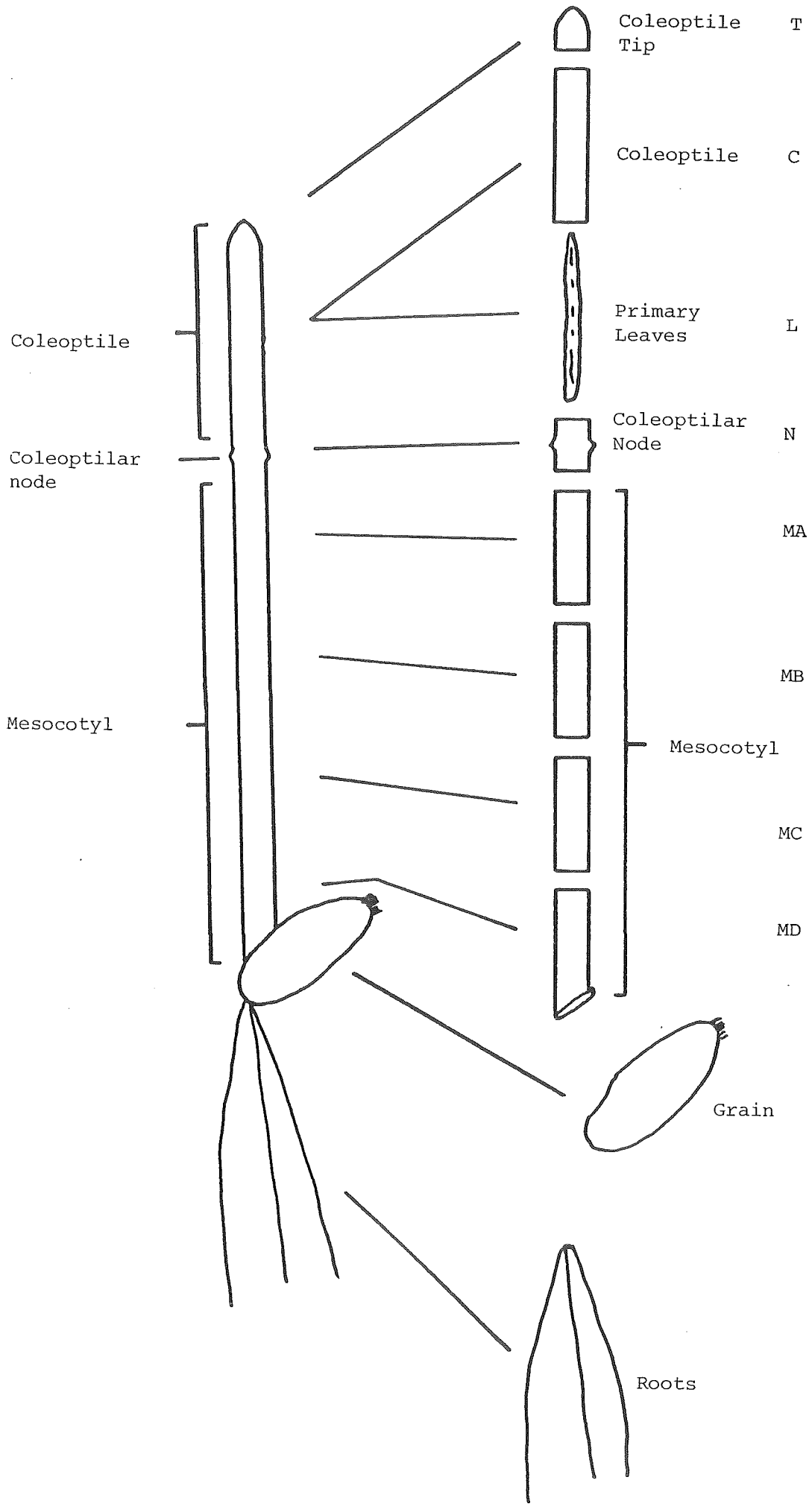
Twenty-five plants were injected for each harvest time. Immediately after injection seedlings, complete in their blotting paper/glass tube assemblies, were placed in 1 dram vials containing 0.5 ml distilled water. The vials were suspended by string in stoppered 25 x 150 mm test tubes. Two ml of 20% aqueous KOH (w/v) was included in the bottom of each test tube to trap evolved $^{14}CO_2$. The seedlings were incubated in the darkroom at 24°C.

2.5.1.2 Harvesting

The labelled plants were harvested in the same sequence in which they were injected. This was particularly important where short incubation times were involved. Seedlings were taken from the darkroom in groups of three, and placed in a refrigerator at 4°C, adjacent to the work area. They were then removed individually and harvested. All manipulations were performed in dim laboratory light.

The plants were sectioned into 10 parts as shown in Fig. 2.1. The radioactivity from each part was extracted and analysed separately. The grain was gently pulled loose at the scutellum, and the roots excised at the junction with the shoot. Using a sharp blade, an incision was made

Figure 2.1: Sectioning of seedling for extraction of radioactivity.


Symbol
Coleoptile Tip
T
Coleoptile
C
Primary Leaves
L
Coleoptilar Node
N
MA
MB
MC
MD
Mesocotyl
Coleoptile
Coleoptilar node
Mesocotyl
Grain
Roots

approximately 2 mm from the apex of the shoot, and the coleoptile tip gently broken loose. Incisions were made 1.5 - 2.0 mm above, and below, the coleoptilar node and the 'nodal segment' (N), 3-4 mm long, removed.

The primary leaves were withdrawn from within the remainder of the coleoptile. These, along with the small amount of leaf material removed from the coleoptile portion of the nodal segment, constituted the leaf fraction (designated L). The remainder of the coleoptile comprised fraction C(Fig. 2.1). The mesocotyl was cut into quarters; designated MA for the apical segment, to MD for the basal, scutellum-containing segment. Approximately 60 min. was required to harvest 25 seedlings.

The lengths of the coleoptile, leaf and mesocotyl segments varied, depending on the time of harvesting after injection. However, the lengths of the coleoptile tip and nodal segments excised from the plant, were kept approximately constant at each harvest time. In a separate experiment, seedlings were grown for 63 h, selected for length, and groups of 25 seedlings were incubated for further periods of 1,3,6, or 24 h. The shoots were harvested as described above, and the fresh weights of the 8 different shoot portions determined for each harvest time. These data were used to express the radioactivity in each segment on a fresh weight basis.

For each harvest, the radioactivity in the blotting paper strips, water reservoir and KOH solution was determined. The reservoir water was poured into one of 5 replicate 20 ml glass scintillation vials, with each scintillation vial receiving the water from 5 seedlings, allocated on a randomised basis. The blotting paper strips from the first 15 seedlings harvested were distributed, in the same order as above, amongst 5 scintillation vials. Similarly the KOH (2 ml) from the test tubes of the first 5 seedlings was transferred to 5 scintillation vials, followed by two 2 ml washes of distilled water. After the addition of scintillation fluid, the radioactivity in each of the above vials was determined by liquid scintillation analysis (LSA, section 2.5.6 below).

2.5.1.3 Extraction

As each seedling was harvested, each part was immediately placed in separate 20 ml vials containing 1.5 ml of chilled (7°C) absolute ethanol. Five replicate sets of vials were used, with 5 segments (or roots or grains) per vial. Seedlings were allocated to one of the replicate series on a randomised basis as for the reservoir water.

Grains were extracted intact, and roots were cut into about 1 cm lengths. Shoot segments were extracted as they were excised from the seedling, except at the 24 h harvest time when the coleoptile segment and the primary leaves were cut into thirds to facilitate handling.

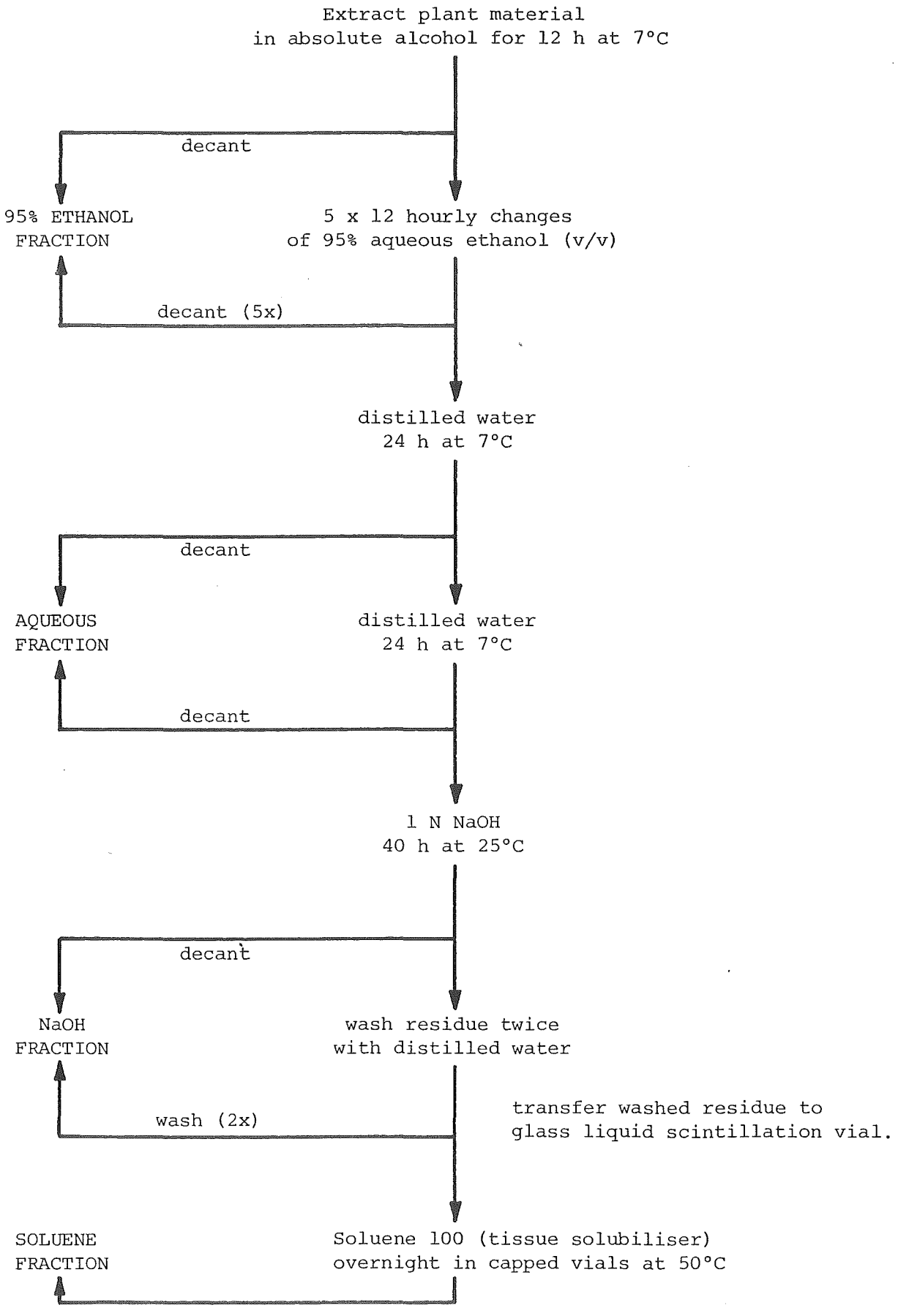
Extraction was in darkness using a modification of Davies' (1976) multiple solvent method as shown in Fig. 2.2. This yielded 4 radioactive fractions; 95% ethanol-soluble, water-soluble, NaOH-soluble and the residue, soluble in Soluene 100 (a tissue-solubiliser, Packard Instrument Co.). The extraction schedule employed 1.5 ml changes of 95% ethanol and 3 ml changes of water per vial. For grains and roots the subsequent extractions in NaOH and Soluene involved 2 ml and 0.5 ml volumes, respectively, whereas for shoot segments, 1 ml NaOH and 0.2 ml Soluene were used. Following NaOH-extraction, the plant tissue was rinsed twice with 1.5 ml distilled water and the rinsings combined with the NaOH extract.

Each fraction was decanted directly into a 20 ml liquid scintillation vial. To prevent inadvertent transfer of segments during decanting, a circle of gauze was fitted to a small glass funnel.

The radioactivity in the aqueous, NaOH and Soluene fractions was determined directly by counting the entire extract by liquid scintillation analysis (section 2.5.6). Half (4.5 ml) of the 95% ethanol extracts of the coleoptile tip, upper quarter of the mesocotyl, and the grain were taken for thin-layer chromatography (TLC). For this, the aliquots from replicates 1 and 2 were combined (solution A), as were the aliquots from replicates 3 and 4 (solution B). Aliquot 5 was divided equally between solutions A and B.

Figure 2.2: Flow diagram for extraction of radioactivity from shoot segments, roots and intact grains

Other details see sections 2.5.1.3 and 2.5.2.


Extract plant material
in absolute alcohol for 12 h at 7°C
decant
95% ETHANOL
FRACTION
5 x 12 hourly changes
of 95% aqueous ethanol (v/v)
decant (5x)
distilled water
24 h at 7°C
decant
AQUEOUS
FRACTION
distilled water
24 h at 7°C
decant
1 N NaOH
40 h at 25°C
decant
NaOH
FRACTION
wash residue twice
with distilled water
wash (2x)
transfer washed residue to
glass liquid scintillation vial.
SOLUENE
FRACTION
Soluene 100 (tissue solubiliser)
overnight in capped vials at 50°C

These solutions (11.25 ml each) were dried by thin-film evaporation under a vacuum of 30 mm Hg at 35°C, and taken up in a few drops of 80% aqueous ethanol (v/v) prior to TLC in MEK/hexane (section 2.5.7.1). After autoradiography (section 2.5.7.2), the radioactivity in the main bands on the TLC plate was quantified by LSA (section 2.5.7.3). TLC was performed at all harvest times in the 2kBq injection experiment, but only at the 6 h harvest in the 200 Bq experiment.

The remainder of the coleoptile tip, upper mesocotyl and grain ethanolic extracts, and the 95% ethanol extracts of the other parts of the seedling, were radioassayed by LSA (section 2.5.6). Where necessary, correction was made for removal of aliquots for TLC.

2.5.2 Injection of 20 Bq IAA-^{14}C

In these experiments 50 plants were used for each harvest time. Seedlings were injected, incubated, and harvested as above (section 2.5.1), but in two batches of 25. This was necessary because 1 h was required to inject 25 plants and thus, for a 1 h incubation time, the first-injected plants had to be harvested immediately after injection of the last plant.

After the first 25 plants were injected, harvested and placed in 3 ml chilled absolute ethanol, the procedure was repeated for the second 25 plants. The plants from both injection sequences were extracted together, with the 50 replicates of each shoot segment in a single series of vials (i.e. there were no replicate extracts of each shoot part). Roots and grains were extracted, 10 items per vial, in 5 replicate vials.

The multiple solvent extraction system as in Fig. 2.2 was used, with 3 ml changes of 95% ethanol and 4 ml of water. All plant material was then extracted in 3 ml of NaOH, with two 2 ml washes of distilled water. The final extraction used 0.5 ml Soluene.

From the 6 h harvest time, half the 95% ethanol extracts of the grain, coleoptile tip and upper quarter of the mesocotyl were taken for TLC and autoradiography (as in section 2.5.1.3).

The radioactivity in the NaOH and Soluene extracts, and in the 95% ethanol solutions sampled for TLC, was counted directly by LSA. The 95% ethanol extracts of those plant parts not sampled for TLC, were concentrated to approximately 6 ml, by thin-film evaporation in 15 x 125 mm test tubes. The contents, including a 2 ml ethanol or water rinse, were transferred to 20 ml scintillation vials and radioassayed by LSA.

As in the 2 kBq and 200 Bq experiments (section 2.5.1), the KOH solution in the test tubes of the first 5 seedlings harvested, and the paper strips from the first 15 seedlings was determined by LSA. The radioactivity in the water reservoir from the first 25 seedlings was also analysed.

2.5.3 Effect of light on IAA-^{14}C transport and metabolism

Sixty seedlings were injected with 20 Bq IAA-^{14}C in the usual manner. Immediately after injection, half the seedlings were exposed to red light for 10 s, and then all the plants were incubated for a further 6 h prior to harvesting. The light-treated and dark-grown seedlings were extracted separately.

Shoots were separated into mesocotyl, coleoptile and primary leaves, and 30 extracted in each vial. The roots were discarded and the grains extracted (10 per vial) in 3 replicate vials. The plant material was extracted as in section 2.5.2. Two separate experiments were performed, on separate days.

Half of the 95% ethanol extracts of the coleoptile, mesocotyl and grain were analysed by TLC, and autoradiographed, as above (section 2.5.1.3). The main regions of radioactivity were assayed by LSA. The activity in the KOH, paper and 1 dram vials was not measured.

A separate experiment was performed to determine the effect of illumination on shoot growth. Sixty three seedlings were selected for uniformity of shoot length at age 63 h. Twenty one of the seedlings were given a 10 s exposure to red light.

Of the remaining seedlings, half were removed from the darkroom for measurement of coleoptile and mesocotyl lengths, and half were incubated in darkness, along with the light-treated plants, for a further 6 h. The lengths of the coleoptile and mesocotyl were then determined, as well as the fresh weights of the coleoptile, mesocotyl and primary leaves of both the darkgrown and illuminated seedlings.

2.5.4 Alternative extraction methods

2.5.4.1 50% acetone

In some later experiments, in which only grains were extracted following injection of 2 kBq IAA-^{14}C, 50% aqueous acetone (v/v) was substituted for 95% ethanol as the organic solvent. In this case, the initial extractant was 52% acetone, with subsequent extractions in 50% acetone, followed by distilled water, NaOH and Soluene as in Fig 2.2, and using the volumes indicated in section 2.5.1.3.

2.5.4.2 Macerated grains

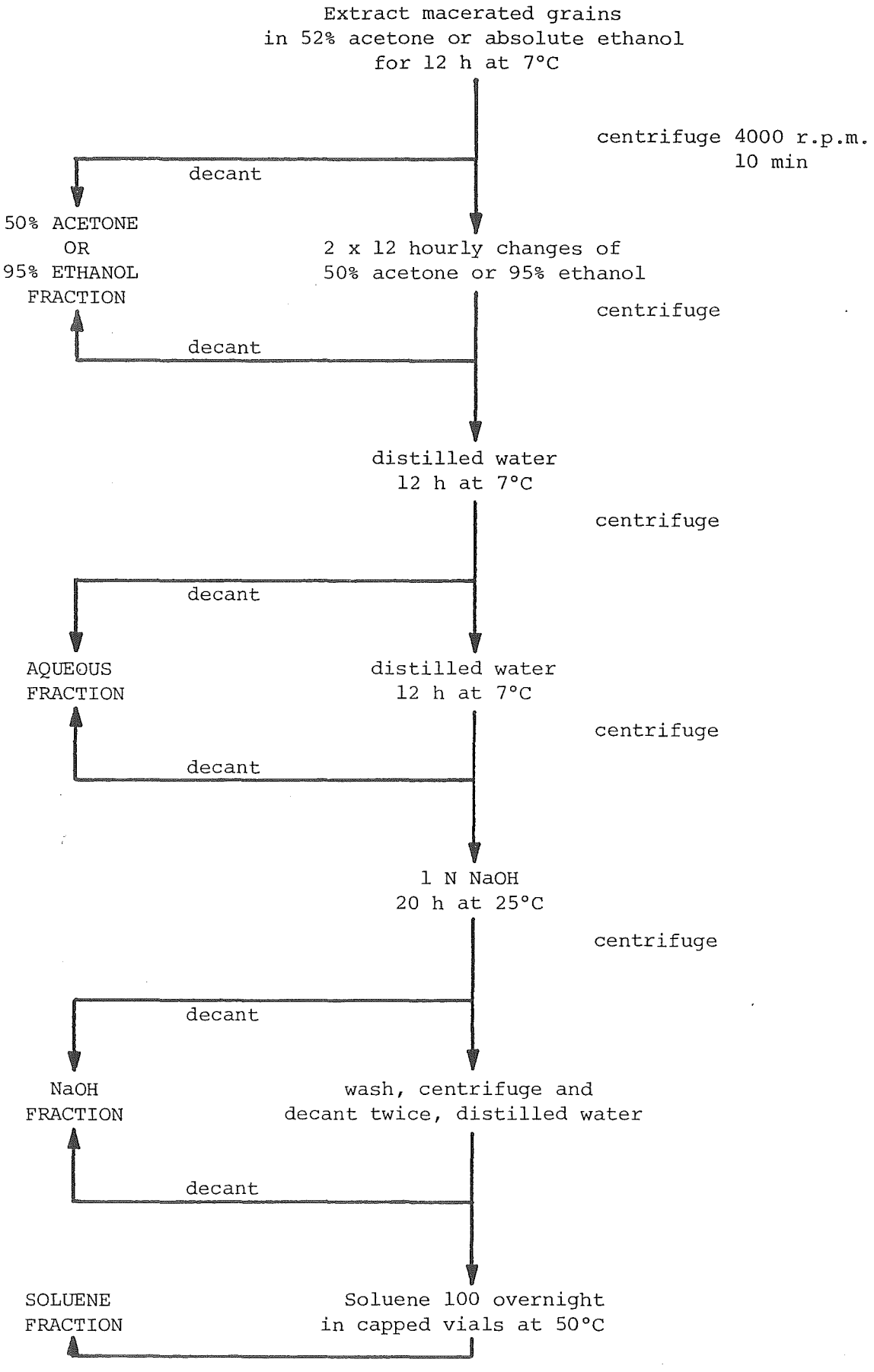
In experiments in which macerated grains were extracted, they were placed in 20 ml vials with the initial solvent [52% aqueous acetone (v/v) or absolute ethanol] and then chopped finely, using fine-pointed scissors, before being ground further with a glass rod. The macerated material was extracted as in Fig. 2.3. For each vial changes of 3 ml organic solvent, 3 ml water, 2 ml NaOH and 0.5 ml Soluene were used.

2.5.5 Acetone precipitation

For preparation of the 'crude ester' fraction the following procedure was used. Ten ml of 50% acetone or 95% ethanol grain extract was centrifuged for 10 min at 4000 r.p.m. (18 cm diam. head). The supernatant was reduced in volume to 0.5 ml of aqueous, by thin-film evaporation, and adjusted to 80% (v/v) with acetone. After thorough agitation, the solution was centrifuged for 10 min at 4000 r.p.m. The supernatant was decanted, concentrated to 0.1 ml and adjusted

Figure 2.3: Flow diagram for extraction of radioactivity from macerated grains

Other details see text section 2.5.4.2


Extract macerated grains
in 52% acetone or absolute ethanol
for 12 h at 7°C
centrifuge 4000 r.p.m.
10 min
decant
50% ACETONE
OR
95% ETHANOL
FRACTION
2 x 12 hourly changes of
50% acetone or 95% ethanol
centrifuge
decant
distilled water
12 h at 7°C
centrifuge
decant
AQUEOUS
FRACTION
distilled water
12 h at 7°C
centrifuge
decant
1 N NaOH
20 h at 25°C
centrifuge
decant
NaOH
FRACTION
wash, centrifuge and
decant twice, distilled water
decant
SOLUENE
FRACTION
Soluene 100 overnight
in capped vials at 50°C

to 90% acetone (v/v). On centrifugation, separation into 2 liquid phases occurred. The upper, colourless phase was decanted. The viscous, dark-brown lower phase was dissolved in water and added to the 80% acetone-insoluble material. This comprised the 'crude ester' fraction.

2.5.6 Liquid scintillation analysis

Liquid scintillation analysis (LSA) was performed using either a Packard Model 2450 or Philips PW4540 liquid scintillation spectrometer operating on the automatic external standard mode.

Data from the Packard instrument were corrected manually for inefficiencies in counting, whereas the Philips spectrometer was interfaced with a Hewitt-Packard 9815 A/S calculator which was programmed to perform this correction automatically. Background counts, which varied depending upon the solvent, were subtracted from the count rates in all cases.

Two scintillation fluids were used: (1) Toluene-Triton X-100 (2:1 v/v), containing PPO (4 $g.l^{-1}$) and dimethyl POPOP (0.1 $g.l^{-1}$) and (2) Toluene containing PPO (4 $g.l^{-1}$).

Solution 1 was employed with all aqueous or water-containing samples (including 95% ethanol extracts), and solution 2 with the Soluene fraction and with other non-aqueous samples. In all cases 10 ml of scintillation solution was used.

2.5.7 Thin-layer chromatography (TLC)

2.5.7.1 Plates and Solvents

Thin-layers (0.25 mm) of silica gel G (Sigma) were prepared on glass according to the manufacturer's directions, except that carboxymethyl cellulose (Whatman CM 32) at 0.3% (w/v) was included in the slurrying water as a gel hardener (Saunders, 1978). Activation, when employed, was in an oven at 100°C for 30 min.

The chromatography solvents used were:

(a) isopropanol/ammonia (0.9l)/water (10:1:1 v/v/v) [iPA].

(b) n-butanol/glacial acetic acid/water (2:5:2.2 v/v/v) [BAW].
(c) chloroform/methanol/water (3:5:2 v/v/v) [$CHCl_3$/MeOH/H_2O]
(d) ethyl acetate/ethanol/formic acid/water (2:5:1:2 v/v/v/v) [EtOAc/EtOH/HCOOH/H_2O].

Buffered silica gel plates were used with the neutral solvent methyl ethyl ketone/n-hexane (35:65 v/v) [MEK/hexane]. These plates were prepared as above, except phosphate-citrate buffer was substituted for water when slurrying the gel. The buffer was made by adding 0.01 M citric acid to 0.02 M Na_2HPO_4, until pH 5.2 was achieved (approximately 4 parts citric acid and 5 parts Na_2HPO_4).

The buffered plates were developed twice for 5 cm, and then twice for 10 cm, with MEK/hexane.

Unless otherwise stated, samples were applied to the origin of the TLC plates as 15 mm wide bands. Plates were developed in darkness at room temperature.

2.5.7.2 Detection of radioactivity

Radioactivity on TLC plates was detected using a Panax model RTLS-1A radio thin-layer scanner coupled to a chart recorder, or by autoradiography, using Kodak X-Ray film or Agfa-Gevaert 'Curix RP2'.

2.5.7.3 Quantification of radioactivity

Where quantification of radioactivity on chromatograms was required following radioscanning or autoradiography, the silica gel in the specified bands was scraped from the plate, into scintillation vials containing 4-6 ml of 50% aqueous ethanol (v/v). After 30 min with occasional agitation, scintillation fluid was added and the radioactivity assayed by LSA.

2.5.7.4 TLC Visualisation agents

Five methods were used to detect compounds on TLC plates.

(i) *Ultra-violet examination.* TLC plates were examined under UV light (360 nm) for any distinctive fluorescence or absorbance.

(ii) *Salkowski reagent.* Indoles were detected by spraying with Salkowski reagent [2.03 g $FeCl_3.6H_2O$ in 200 ml distilled water and 300 ml conc. H_2SO_4 (specific gravity 1.840)], and heating the plates at 105°C for 5-10 min.

(iii) *Aniline-Diphenylamine reagent.* To detect sugars, two solutions were prepared. These were sprayed onto the plate sequentially and it was heated as above. Solution 1 consisted of 2 ml aniline and 2 g diphenylamine made up to 100 ml with acetone. Solution 2 was 20 ml phosphoric acid (85%) diluted to 100 ml with acetone.

(iv) *Concentrated Sulphuric Acid.* Charrable material was detected by spraying TLC plates with conc H_2SO_4 and heating to 105°C.

(v) *Ninhydrin reagent.* Amino acids react to produce either an orange or purple colour when heated briefly with ninhydrin (0.2% in acetone, w/v). The reagent was prepared freshly before use.

2.5.8 Column chromatography

2.5.8.1 Materials

Some grain extracts were purified by Sephadex (Pharmacia) gel filtration. A range of gels (G-15, G-25 and G-50) and column sizes was used.

Gels were soaked for at least the recommended period in distilled water and deaerated prior to preparation of the column.

Samples applied to the column were eluted with distilled water and 2 ml fractions collected, after elution of the void volume (determined using blue dextran, Pharmacia). In some experiments, elution patterns were monitored using an ISCO model UA-4 UV monitor, set at 280 nm.

2.5.8.2 Assay procedures

A number of assays were used to detect compounds eluting from the Sephadex columns.

(i) *Carbohydrates.* Carbohydrate was quantified using the anthrone test. Four ml of 0.2% anthrone in conc H_2SO_4 (specific activity 1.840) (w/v) was syringed rapidly onto 0.5 ml of aqueous sample in 16 x 100 mm screw cap culture tubes (Sovirel). The tubes were sealed with teflon-lined caps, thoroughly mixed and placed in a water-bath at 45°C for 15 min to dissipate the reaction heat. After further cooling in running tap water, the absorbance of the solutions was measured directly in the culture tubes, using a WPA CO 65 colorimeter with a No. 6 filter. The amount of carbohydrate was expressed in glucose equivalents after comparison with a standard curve.

(ii) *Indoles.* Indoles were determined with Ehmann reagent (Percival and Bandurski, 1976). This reagent consisted of 3 parts Salkowski reagent (section 2.5.7.4) and 1 part Ehrlich reagent (v/v). For Ehrlich reagent, 1.25 g p-dimethylaminobenzaldehyde (Eastmans), decolourised with activated carbon and recrystallised from ethanol-water (m.p. 74.5°C), was dissolved in 50 ml conc H_2SO_4 (specific gravity 1.840) and 50 ml absolute alcohol was added. This reagent was stored in a brown glass bottle in darkness at 4°C.

For the Ehmann assay, samples were dried, dissolved in 10 drops of 80% aqueous ethanol (v/v), and 0.4 ml of reagent was added. Mixtures were heated at 45°C for 60 min, cooled and diluted with 1 ml of distilled water. Absorbance was measured in a 12 x 100 mm colorimeter tube, using the WPA CO65 colorimeter with a No. 5 filter. The results were expressed in IAA equivalents by comparison with a standard curve.

(iii) *Peptides.* Biuret reagent was prepared by dissolving 0.3 g of $CuSO_4.7H_2O$ and 0.9g of sodium potassium tartrate in 50 ml of 0.2 N NaOH, and adding 0.5 g of KI.

The solution was made up to 100 ml with 0.2 N NaOH. For the assay of peptides, 4 ml of Biuret reagent was added to 1.5 ml of sample, and the solutions were mixed and warmed at 37°C for 10 min. After cooling, the absorbance was measured colorimetrically (No. 5 filter). Protein standards were prepared freshly, using albumin at concentrations of 4 to 20 $mg.l^{-1}$.

As a qualitative test for the presence of amino groups, 10 drops of freshly prepared 0.2% aqueous ninhydrin (w/v) were added to 1 ml aliquots of sample and the solutions boiled for 5 min to allow colour development, which was assessed visually.

2.6 STUDIES ON THE EFFECT OF UNLABELLED GROWTH REGULATORS

2.6.1 Injection into the endosperm

In separate experiments, solutions of IAA, GA_3 ABA or kinetin were injected into the endosperm of 63 h old seedlings which had been selected for uniformity of height (section 2.3).

In each of these experiments, the test solutions were: growth regulator at 2.5×10^{-3}, 2.5×10^{-4} and 2.5×10^{-5} M, and a control solution lacking growth regulator (prepared as in section 2.4). The experimental procedure was as follows. Five seedlings were injected with one of the test solutions (i.e. concentrations). Further groups of five were then injected with the other 3 test solutions. The order in which the solutions were applied was randomised. This injection procedure was repeated 3 more times, and again the order of solutions was randomised. Thus for each test solution, 4 batches of 5 seedlings were injected, making a total of 80 seedlings per experiment. The experimental design was 'split plot' (Snedecor and Cochran, 1967) with each random sequence of application of test solutions representing a 'block', separated in time from other blocks.

Immediately after injection, the seedlings were returned to their growth containers and incubated in darkness at 24°C for a further 24 h, after which the lengths of the roots, mesocotyl and coleoptile were measured to the nearest millimetre. Values were expressed as the mean length of 20 seedlings $\pm$ the standard error. In some experiments, the effect of injection on seedling growth was assessed, by selecting 63 h old seedlings and incubating them for 24 h without further treatment.

To test the effects of growth regulators on plants exposed to light, identical experiments were performed, except that after injection of each 'block' of seedlings (i.e. each random sequence of test solution injections), the plants were exposed to red light for 10 seconds (section 2.2.2).

2.6.2 Application of IAA to the coleoptile tip

The experimental design and procedure of section 2.6.1 was employed in experiments where the effect of IAA applied

to the coleoptile tip in agar blocks was studied. The test blocks contained IAA at 2.5×10^{-3} M, 2.5×10^{-4} M or 2.5×10^{-5} M, and the control was agar without IAA (prepared as in section 2.4). In some experiments, as an additional control, seedlings were selected for length 63 h after planting and incubated without agar blocks.

Both dark-grown and light-treated plants were tested, and, as in section 2.6.1, the seedlings were measured 24 h after treatment was administered.

2.6.3 Histological studies

Where the effects of growth regulators and light on cell elongation and extension in the mesocotyl and coleoptile were studied, separate experiments were performed to provide the plant material for the histological studies. The three plants with coleoptile and mesocotyl lengths most closely approximating the mean treatment length were selected. Each shoot was cut into approximately 10 mm lengths for convenience of handling. Segments were fixed in formalin/acetic acid/alcohol (FAA), progressively dehydrated in alcohol, and infiltrated and embedded in parafin (Jensen, 1962). Sections 10 μm thick were cut, longitudinally and parallel to the plane of symmetry of the seedling, using a Jung rotary microtome. The sections were stained with safranin-fast green and mounted using 'Depex'. The slides were viewed on a Riechert 'Visapan' microscope.

Using median sections, the number of cells per millimetre, in each of 3 separate columns was counted (in the cortex of the mesocotyl; the subepidermis and 3rd and 4th layers in the coleoptile). This was done at millimetre intervals along the length of both structures, and the mean number of cells per column computed. The variation in cell length along the coleoptile and mesocotyl was derived by taking the reciprocal of the mean number of cells per millimetre. Since 3 replicate plants were sectioned from each sample, the mean number of cells per column was averaged to produce a grand mean.

2.7 ANALYSIS OF GASES IN GROWTH CONTAINERS

Lengths of copper tubing (5 mm internal diameter) were inserted through rubber grummets fitted in the lid of growth containers, so that gas samples could be taken from the region in which the seedlings were growing. The tubes were purged by vacuum prior to sampling.

2.7.1 Carbon dioxide

CO_2 was measured using an Orsat apparatus, in which a 100 ml sample of air is taken, and the CO_2 level determined by the change in volume, after absorption of the CO_2 in KOH.

2.7.2 Ethylene

Ethylene was determined with a Tracor model 550 gas chromatograph (GC), using flame ionisation detection. The following columns and conditions were used:

Column A - 100 mesh deactivated alumina, 1.22 m x 3.2 mm.
N flow - 50 ml. min^{-1}
oven - 90°C
detector - 270°C
injection port - 120°C
H_2 flow - 40 ml.min^{-1}

Column B - Porapak Q, 80-100 mesh, 1.7 m x 3.2 mm
N flow - 50 ml.min^{-1}
oven - 65°C
detector, injection port and H_2 flow as above.

Two ml gas samples from the growth containers were injected using a Glenco gas-tight syringe. The GC was calibrated using ethylene standards mixed with air, ranging from 0.1 p.p.m. to 50 p.p.m. ethylene. The standards were prepared in glass flasks fitted with rubber septa.

2.8 STATISTICAL ANALYSIS

Generally, means and standard errors of the means were computed for all seedling length measurements and for the data derived from the radioactive-labelling experiments.

The significance of differences between means was determined using the Student's t-test, or the least significant difference method (LSD) in conjunction analysis of variance (Anova) (Sokal and Rohlf, 1969). For the unlabelled growth regulator experiments, 'split plot' analysis of variance was performed using the 'Teddy-bear' programme (Univ. of Otago, 1979), on a Burroughs B6700 computer.

Data from the light vs. dark IAA-^{14}C experiment (section 2.5.3) were analysed using the Chi-square 'goodness of fit'.

Vertical bars indicated on graphs represent twice the standard error (s.e.) of the mean.

3.0 RESULTS

3.1 EVALUATION OF GROWTH CONDITIONS

3.1.1 Growth containers

3.1.1.1 Determination of carbon dioxide and ethylene levels

The accumulation of gaseous by-products of plant growth is often a problem in experiments carried out in enclosed chambers. To test the effectiveness of the ventilators on the growth containers used in this investigation, air samples were collected at regular intervals from canisters containing 90 growing seedlings. These samples were assayed for carbon dioxide and ethylene. No accumulation of either compound was detected even after 7d incubation. For ethylene, this meant the concentration in the containers was less than 0.1 p.p.m., which was the lowest level detectable by the gas chromatograph, and was similar to the amount occurring in normal laboratory air. The Orsat Apparatus used for determining carbon dioxide levels had a minimum sensitivity of 0.1% (v/v). As the normal concentration of CO_2 in air is 0.03%, more than a threefold increase could have occurred before carbon dioxide was detected in the chambers.

3.1.1.2 Bioassay of gaseous products

Growth of oat seedlings was used as a more appropriate test of gaseous accumulation. In Fig. 3.1 the results are shown of an experiment in which seedlings were grown in growth containers at different densities. In one series of chambers ventilators were blocked to prevent aeration, while in another the ventilators remained open. After 7d growth, the mean mesocotyl and coleoptile length was determined from a random sample of 40 plants taken from each chamber.

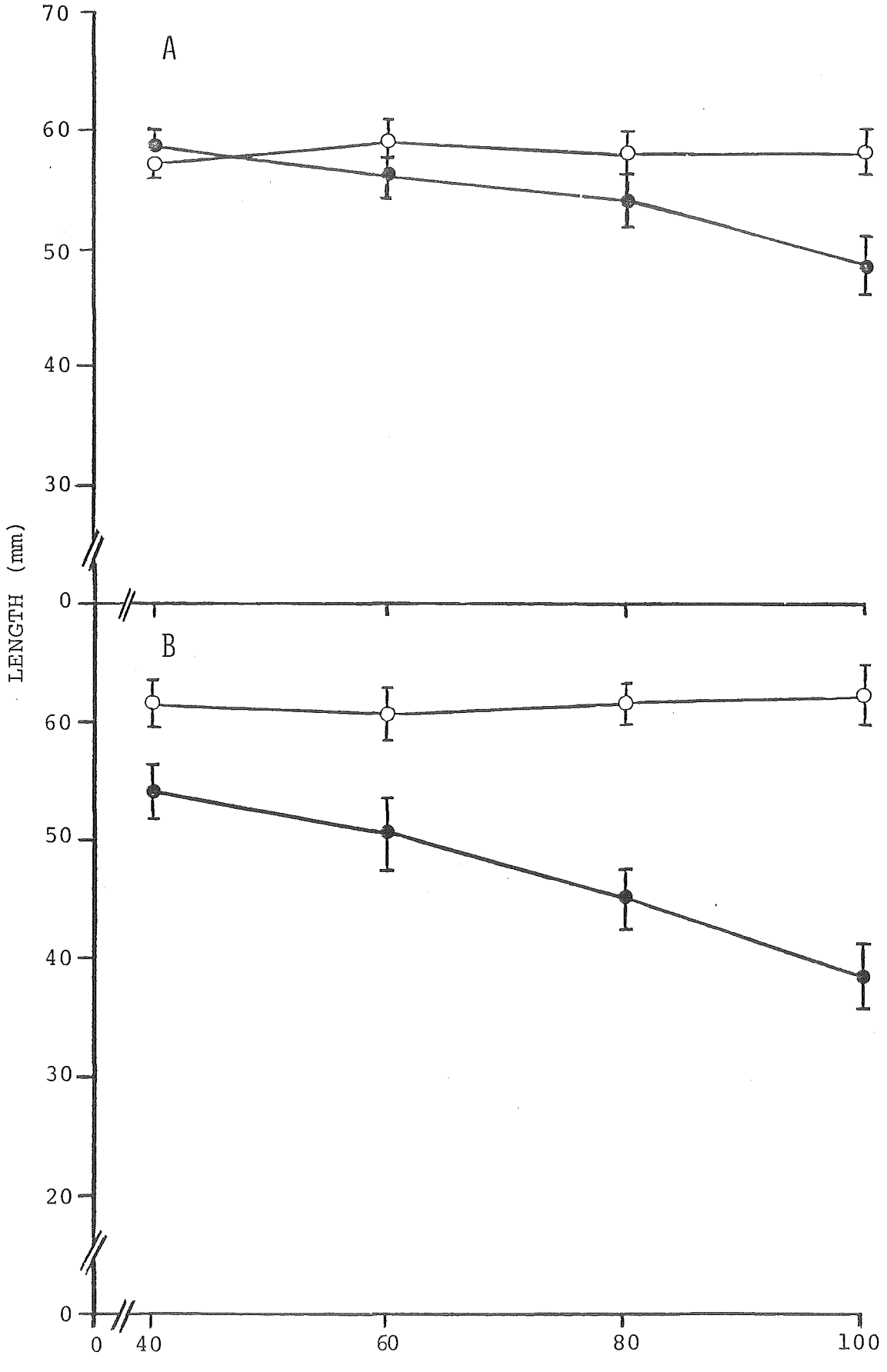
No significant difference in the length of the shoot was observed between any of the plant densities, when ventilators remained open (Anova, $P < 0.05$). When chambers were not ventilated, the elongation of both the coleoptile

Figure 3.1: Effect of seedling density in growth containers on elongation of (A) the coleoptile and (B) the mesocotyl

Mean of 40 measurements ± standard error.

○ Ventilators open

● Ventilators closed


A
B
70
60
50
40
30
0
60
50
40
30
20
0
0
40
60
80
100
LENGTH (mm)
NUMBER OF SEEDLINGS PER CONTAINER

(Anova, $P < 0.05$) and mesocotyl (Anova, $P < 0.01$) was inhibited. The degree of inhibition increased with higher seedling density and was accompanied by an accumulation of carbon dioxide in the growth containers, proportional to the number of seedlings present (Fig. 3.2). No accumulation of ethylene was observed. It was found that a similar reduction in coleoptile and mesocotyl length could be induced by administering exogenous carbon dioxide to growing seedlings (Table 3.1).

Table 3.1: Effect of exogenous CO_2 on shoot elongation

Forty hours after imbibition, ventilators of 2 growth containers (containing 60 seedlings) were blocked, and CO_2 applied to give a concentration of 1.5% in one container, and 3.0% (v/v) in the other (monitored using Orsat apparatus). The containers were opened and purged with air every 12 h, after which CO_2 was reapplied. A control container of seedlings with ventilators open and no added CO_2 was maintained. Mean mesocotyl and coleoptile lenghts were determined at the end of 7 d.

For each structure, means which are not significantly different (LSD, $P < 0.01$), are designated with a similar subscript.

Treatment	Mean length (mm)	
	Coleoptile	Mesocotyl
Control (no CO_2)	59.8 a	62.1 d
1.5% CO_2	58.1 a	48.9 e
3.0% CO_2	49.3 b	38.3 f

3.1.2 Effects of 'safelights'

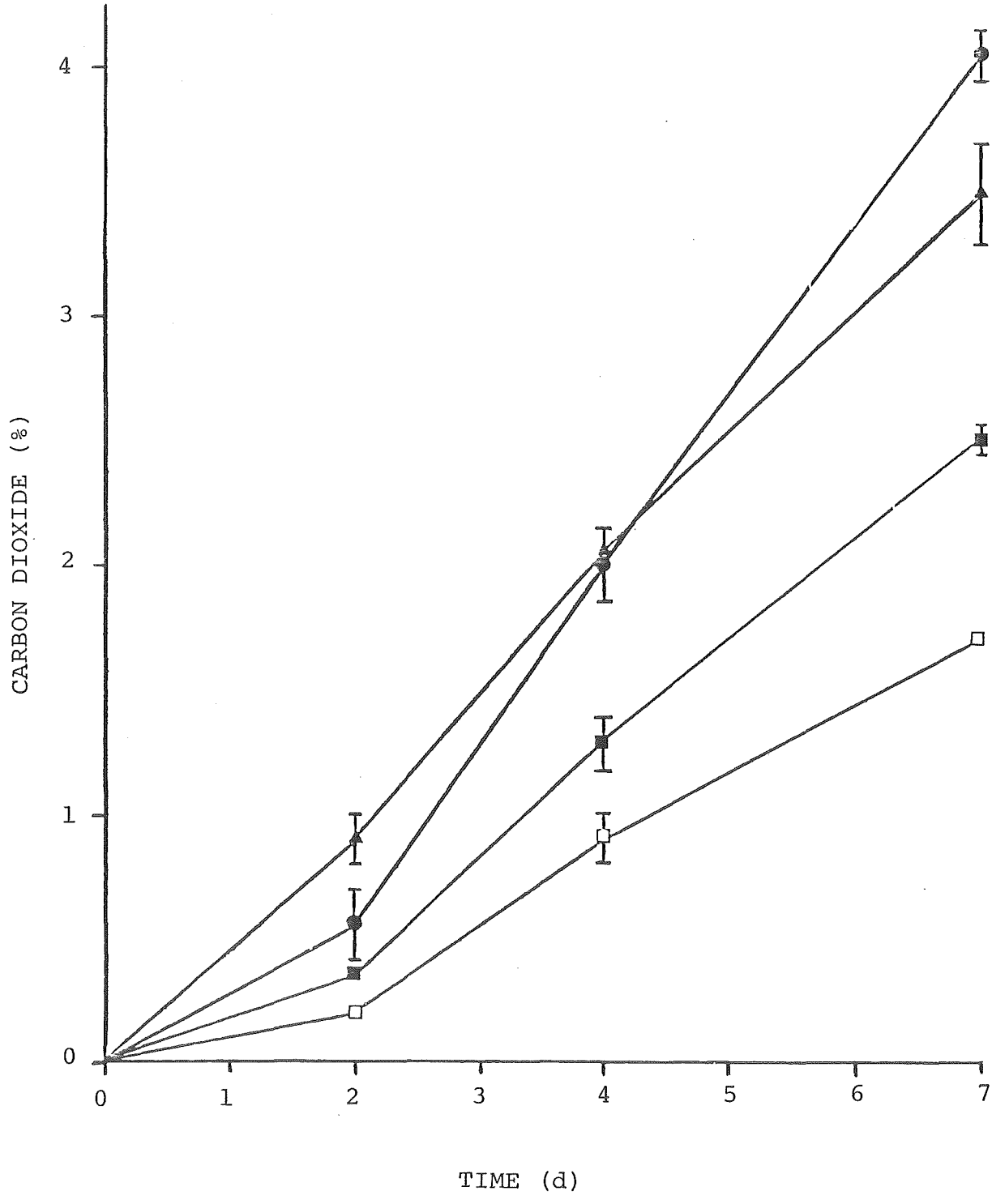
The term safelight is misleading since all wavelengths of light in the visible spectrum have been shown to have an inhibitory effect on mesocotyl growth (Weintraub and McAlister, 1942). Therefore, the use of 'safelights' should be avoided

Figure 3.2: Accumulation of CO_2 in unventilated growth containers

(n = 2)

● 100 seedlings per container
▲ 80 seedlings per container
■ 60 seedlings per container
□ 40 seedlings per container

N.B. Where no standard error is shown the error term is less than or equal to the size of the symbol used to designate the mean.


CARBON DIOXIDE (%)
0
1
2
3
4
0
1
2
3
4
5
6
7
TIME (d)

in experiments which involve the study of etiolated growth of Avena seedlings. In practice, however, this is not always possible since certain manipulations require the aid of a 'safelight'. The darkroom used in this study was equipped with two green 'safelights'. Their effect on seedling development was investigated.

3.1.2.1 Individual lights

Groups of 63 h old seedlings (not selected for uniformity of length) were given 5 min exposure, either to the individual 'safelights' (large or small, see section 2.2.1), or 5 min exposure to both lights together. Mesocotyl, coleoptile and root lengths were measured after a further 4 d incubation in darkness (Table 3.2).

Table 3.2: Effect of 'safelights' on seedling extension

For details see text. For each structure, means which are not significantly different (LSD, $P < 0.01$), are designated with a similar subscript. (n = 35 - 37).

Treatment	Mean length (mm)		
	coleoptile	mesocotyl	root
Control (no light)	64.7 a	63.7 b	73.8 d
Large 'safelight' (5 min)	65.1 a	53.2 c	71.2 d
Small 'safelight' (5 min)	67.1 a	52.9 c	70.7 d
Both 'safelights' together (5 min)	63.5 a	53.7 c	72.3 d

Coleoptile and root elongation was unaffected by exposure to the 'safelights', but a reduction in mesocotyl extension was observed. The two 'safelights', when used separately, resulted in similar amounts of mesocotyl inhibition; and concurrent exposure to both lights did not

cause a further reduction in growth. On average, light-treated mesocotyls were 16% shorter than their dark-grown counterparts.

The experiment was repeated twice more and all 3 results confirmed that while mesocotyl elongation was inhibited by the 'safelights', coleoptile and root extension was unaffected.

3.1.2.2 Length of exposure

The effect of length of exposure was investigated using both 'safelights' together. As before, plants were grown in darkness for 63 h and then exposed to the 'safelights'. After a further 4 d, mesocotyl, coleoptile and root lengths were measured (Fig. 3.3). Inhibition of mesocotyl elongation was found to increase as the duration of exposure to the 'safelights' was extended. The response appeared to be saturated at the higher exposures tested; doubling the exposure time from 5 to 10 minutes did not induce further reductions in mesocotyl length beyond the 16% inhibition observed previously (Table 3.2, above). One minute exposure did not significantly affect the final mesocotyl length, but the 11.4% inhibition that resulted from the two minute exposure, was statistically significant (LSD, $P < 0.01$).

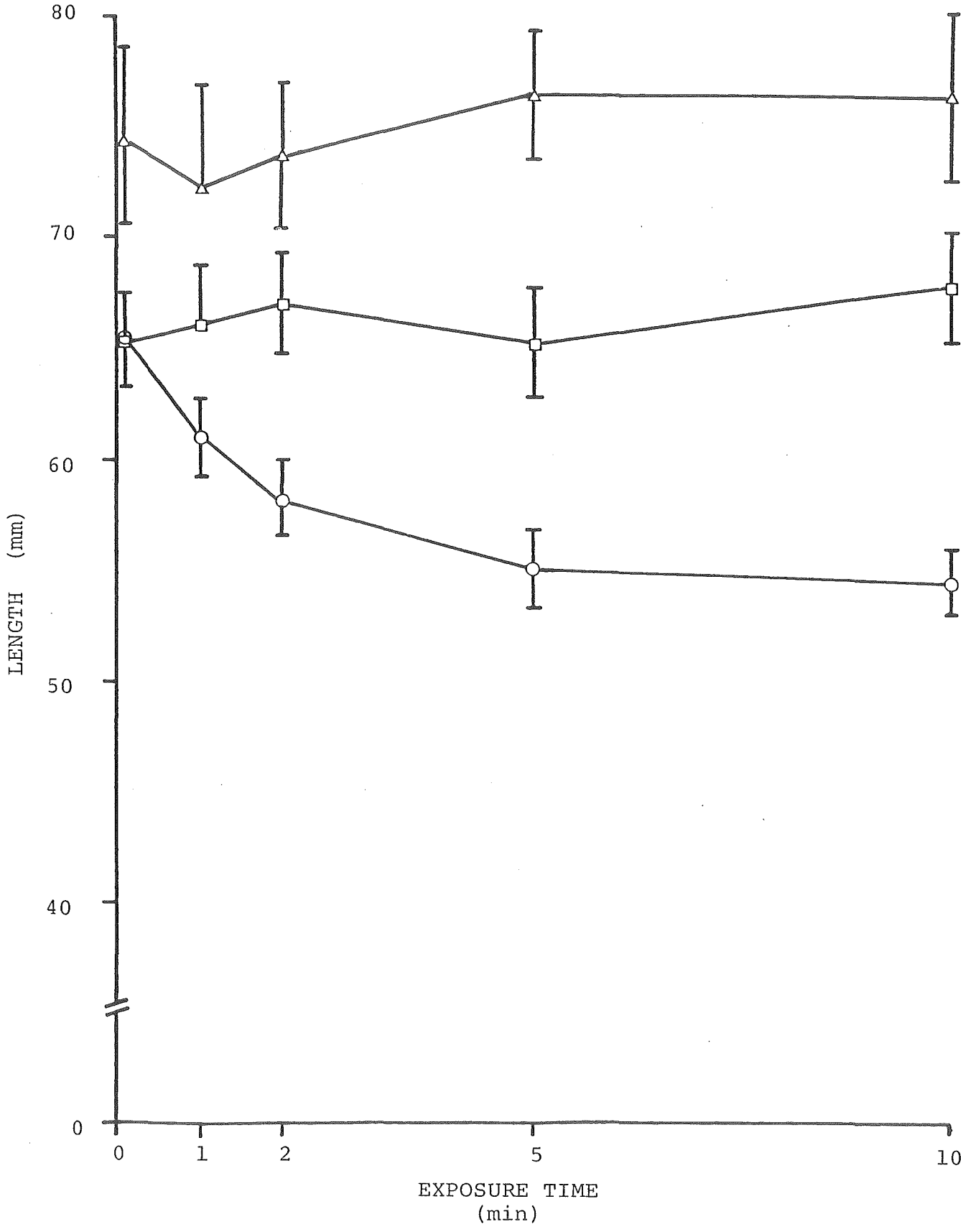
None of the exposure times tested significantly affected coleoptile or root elongation.

On the basis of these results it was decided that where use of 'safelights' proved necessary, care should be taken to ensure that all seedlings receive uniform exposures of light. The following procedure was adopted for later experiments; both safelights were used, seedlings were removed from the light-proof growth containers only when required, and the exposure time of seedlings to 'safelights' was kept to between 5 and 10 min (i.e. on the flat part of the mesocotyl response curve).

Figure 3.3: Effect of different 'safelight' exposures on seedling extension growth

For details see text (n = 35 - 38).

○ Mesocotyl
□ Coleoptile
△ Roots


80
70
60
50
40
0
LENGTH (mm)
0
1
2
5
10
EXPOSURE TIME
(min)

3.2 SEEDLING DEVELOPMENT

3.2.1 Growth in darkness

The typical pattern of shoot development in dark-grown oats (cv. 'Terra') is shown in Fig. 3.4. Both the coleoptile and mesocotyl exhibited characteristic sigmoid growth curves, but with elongation maxima of different rates and at different times.

In both structures, only a small amount of elongation occurred in the first 2 days after imbibition started, with slightly more extension taking place in the mesocotyl. During the third day, the mesocotyl elongated considerably, to more than double the length of the coleoptile. Rapid extension of the mesocotyl continued throughout the fourth day, after which the growth rate declined, and by the end of the sixth day after planting, the final mesocotyl length of approximately 65 mm, had been achieved.

The coleoptile, which entered its phase of rapid growth 18 to 24 h after the mesocotyl, elongated at a slower rate than the mesocotyl and the decline in the rate of extension was less well defined. By the end of the experimental period (9 d), the coleoptile was approximately 70 mm long and elongation appeared to have virtually ceased.

Crown roots appeared in the region of the coleoptilar node on some plants during the sixth day after planting, and by the end of the seventh day these were present on all plants. At this stage, as extension growth of the coleoptile declined, the first leaves, carried up by the elongating 'second' internode, broke through the tip of the coleoptile. After 8 d, the 'second' internode protruded from the top of the coleoptile.

3.2.2 Growth after red light treatment

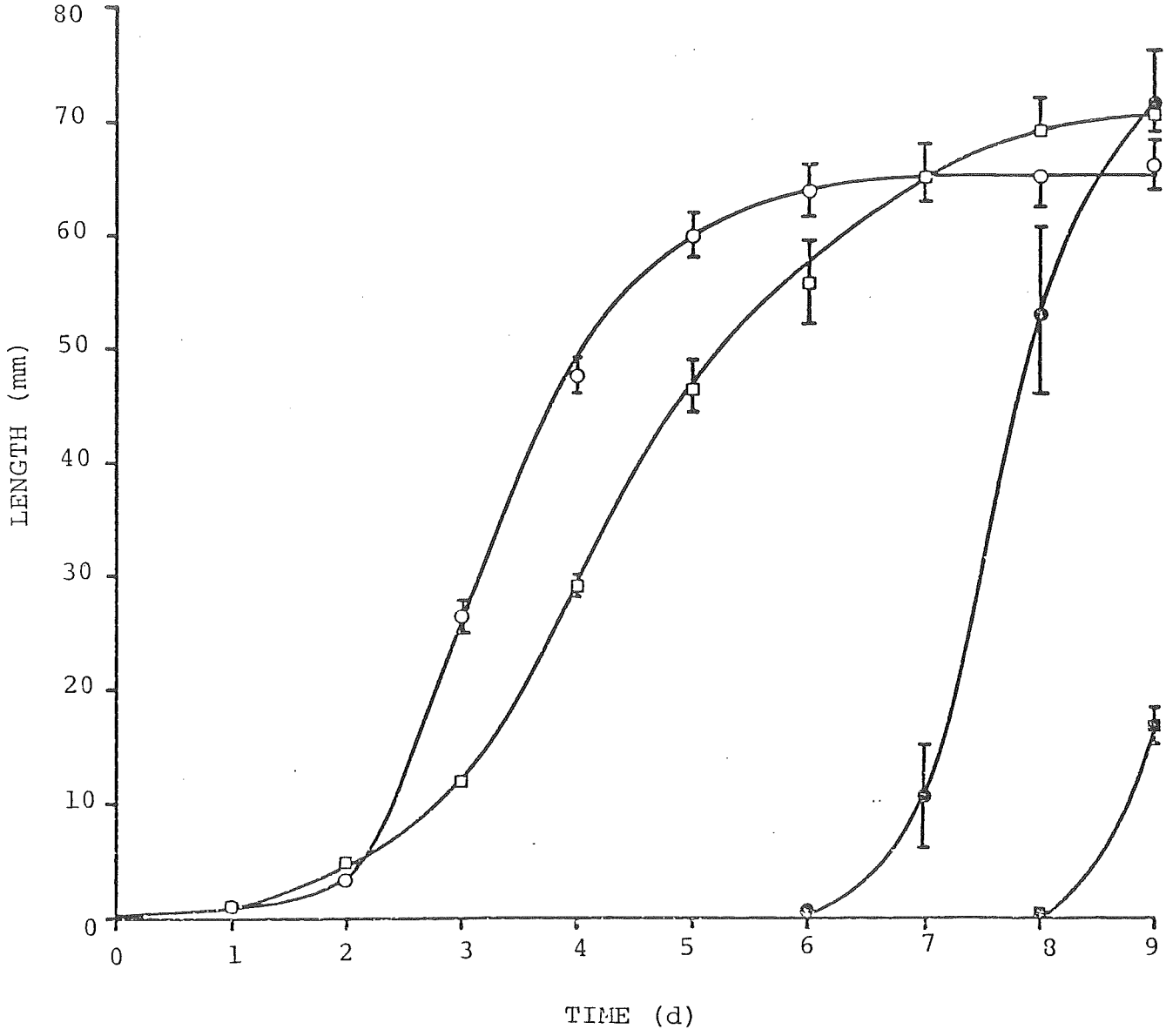
The effect of a brief exposure to red light on seedling development was investigated (Fig. 3.5).

The plants were exposed for 10 s, 63 h after imbibition commenced. The most obvious result was an almost immediate cessation of mesocotyl elongation. In addition, the growth pattern of the coleoptile was altered, so that it resembled that of the

Figure 3.4: Shoot extension in darkness

Values represent mean ± standard error of 35 - 37 plants.

○ Mesocotyl
□ Coleoptile
● First leaf
■ 'Second' internode


80
70
60
50
40
30
20
10
0
LENGTH (mm)
0
1
2
3
4
5
6
7
8
9
TIME (d)

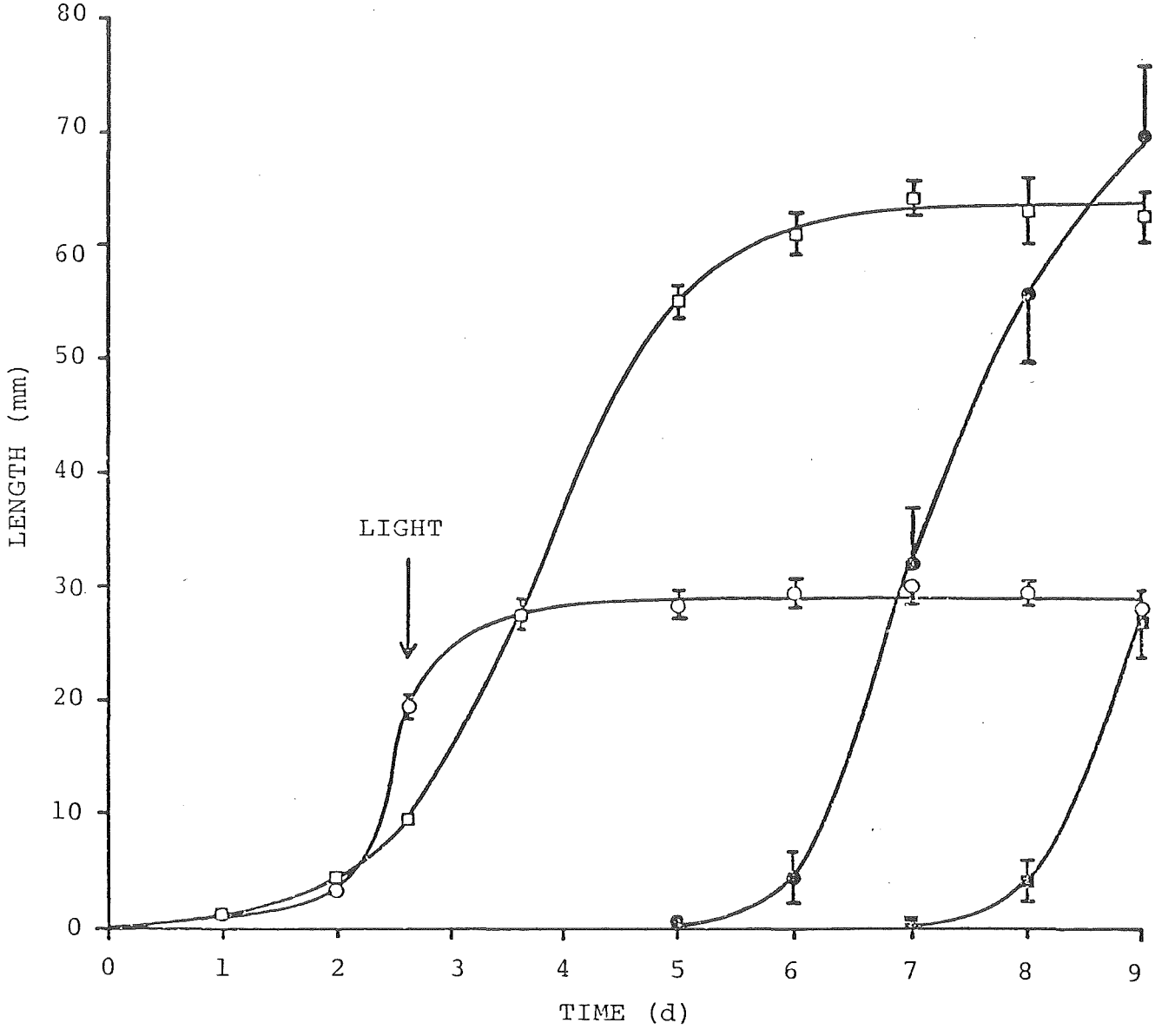
dark-grown mesocotyl (Fig 3.4, above), with a steeper phase of rapid elongation and a more abrupt and earlier end to extension growth.

Although the rate of elongation of the coleoptile was accelerated by light (mainly during the 5th day after planting), the earlier cessation of elongation meant that, ultimately, light-treated coleoptiles were shorter than their dark-grown counterparts. Light treatment also resulted in the earlier appearance of crown roots, the primary leaves and the 'second' internode.

Figure 3.5: Shoot extension in darkness with brief light interruption

Values represent mean ± standard error of 35-36 plants.

○ Mesocotyl
□ Coleoptile
● First leaf
■ 'Second' internode


80
70
60
50
40
30
20
10
0
LENGTH (mm)
LIGHT
0
1
2
3
4
5
6
7
8
9
TIME (d)

3.3 RADIOTRACER STUDIES

3.3.1 IAA-^{14}C stability

Preliminary experiments involved the development of a technique for injection of IAA-^{14}C into the endosperm; optimisation of seedling incubation times; as well as the establishment of a suitable extraction and purification method.

3.3.1.1 Breakdown of IAA-^{14}C on TLC plates

During the course of some early experiments, radioactive 'impurities' were noted during TLC of IAA-2-^{14}C standards. The main 'impurity' ran to Rf 0.30-0.38 in MEK/hexane (Fig. 3.6a), and in iPA and BAW, ran to Rf 0.60 - 0.75 and 0.62 - 0.70 respectively (Fig. 3.6 b and c)(for specifications and abbreviations see section 2.5.7).

The 'impurities' were present on chromatograms, even when IAA-^{14}C was sampled directly from the supplier's vial, without being subjected to manipulations such as evaporation under reduced pressure. This suggested that the stock solution itself may have undergone decomposition, despite being stored in darkness at -10°C. Since the IAA-^{14}C solution was a number of months old, a new batch was obtained from the manufacturer. This was immediately tested for purity. Special precautions were taken when the vial was opened and sampled, with all manipulations being performed under an N_2 atmosphere in dim incandescent lighting. An aliquot of the IAA-^{14}C solution was analysed by TLC in MEK/hexane and the radioactivity located using a radiochromatogram scanner (Fig. 3.7). The 'impurity' was also evident with the new IAA-^{14}C.

The manufacturers claimed 99.8% purity of the radioactive IAA and, given the precautions taken during sampling of the solution, this suggested that destruction was occurring on the chromatogram - either prior to, or during, development of the plate.

Figure 3.6: Autoradiographs of chromatograms of IAA-^{14}C and 'impurities' developed in different solvent systems

(O = Origin, F = Solvent Front)

A. MEK/hexane

B. BAW

C. iPA

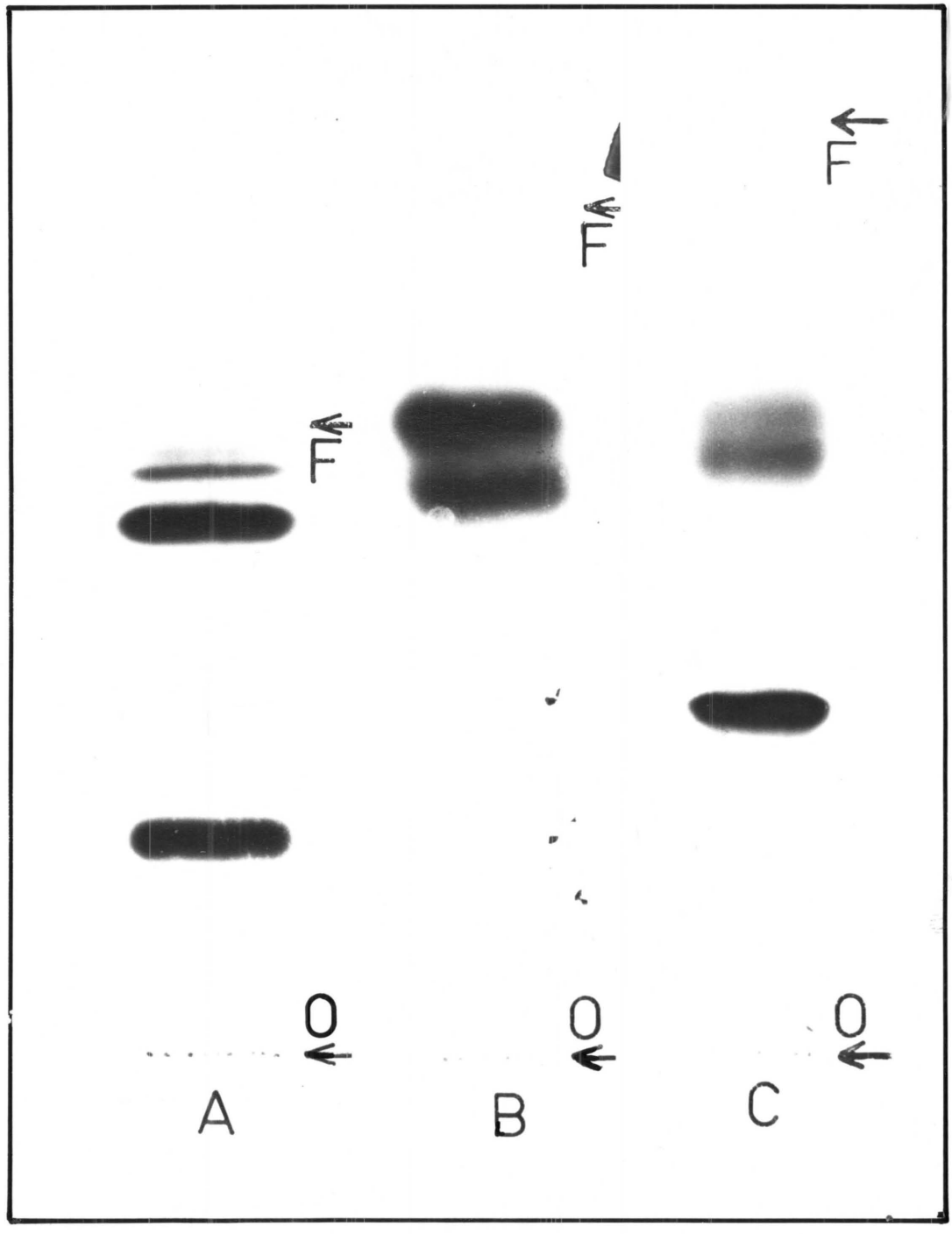
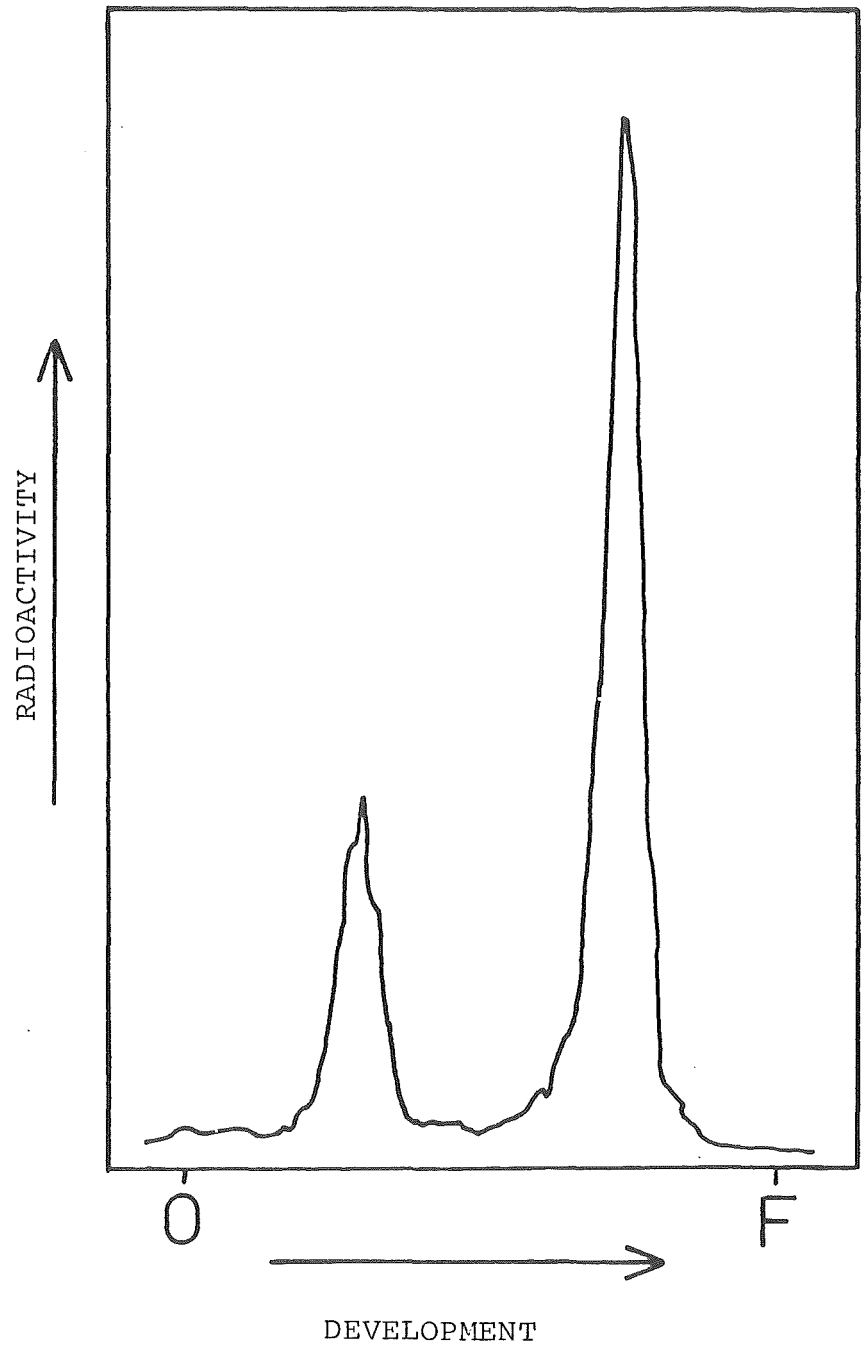

F
F
F
O
O
O
A
B
C

Figure 3.7: Radioscan tracing of silica gel chromatogram of new IAA-^{14}C spotted in dim light under nitrogen, and developed in MEK/hexane

(O = Origin; F = Solvent Front)


RADIOACTIVITY
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DEVELOPMENT

(i) *Induction of* IAA-^{14}C *breakdown.* Mann and Jaworski (1970) reported that IAA was oxidised during application of solutions to the origin of chromatograms, but that the chromatography solvent itself did not cause immediate destruction of IAA. Experiments were carried out to determine whether IAA-^{14}C was decomposed during TLC , and if so, at what stage of the procedure this was occurring.

Buffered silica gel plates (5 x 20 cm) were spotted under N_2, with 115 Bq IAA-^{14}C dissolved in methanol. The TLC plates were developed in MEK/hexane, either immediately or after an interval. At the same time, the possible effect of light on IAA breakdown was studied. One set of the chromatography plates spotted with IAA-^{14}C was maintained in darkness, while another was exposed to light from a 60 W incandescent lamp at a distance of 50 cm. The plates were incubated at 24°C. Radiochromatogram scans of the developed plates are shown in Fig. 3.8. Only IAA-^{14}C was apparent on plates that were developed immediately after spotting. Any delay in development of the spotted plates resulted in IAA destruction and the formation of labelled products, the main one of which ran to Rf 0.22-0.35.

In darkness, formation of the breakdown products was slow, and even after 60 min incubation prior to development, only about 10% of the applied IAA-^{14}C was in this form. In contrast, in light, after the same period, approximately 35% of the total activity was accounted for in the decomposition products.

(ii) *Breakdown under normal laboratory conditions.* Additional experiments were carried out to determine the extent of IAA-^{14}C destruction under normal laboratory lighting conditions i.e. natural (summer) daylight supplemented with white fluorescent lighting.

IAA-^{14}C (50 Bq) was applied under N_2 in 15 mm wide bands to the origin of duplicate TLC plates (20 x 20 cm). Each band was applied at a different interval prior to development of the plate, and immediately after application of the final band, the plates were developed in iPA. The

Figure 3.8: Time-course of IAA-^{14}C breakdown on silica gel TLC plates in presence or absence of light.

The developing solvent was MEK/hexane. The number in the top left-hand corner of each radioscan signifies the period the plate was incubated prior to development.

N.B. Figure 3.8 continued on following page.
For other details see text.

(O = Origin; F = Solvent Front)

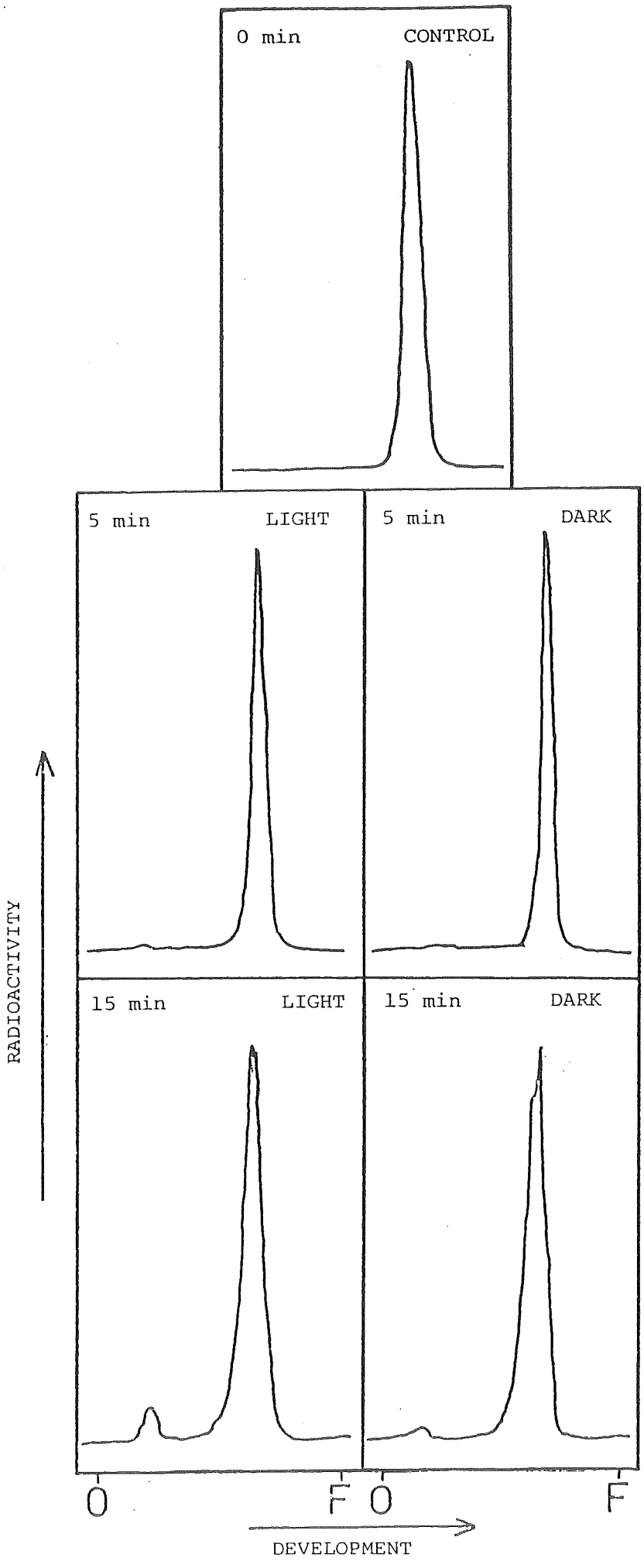
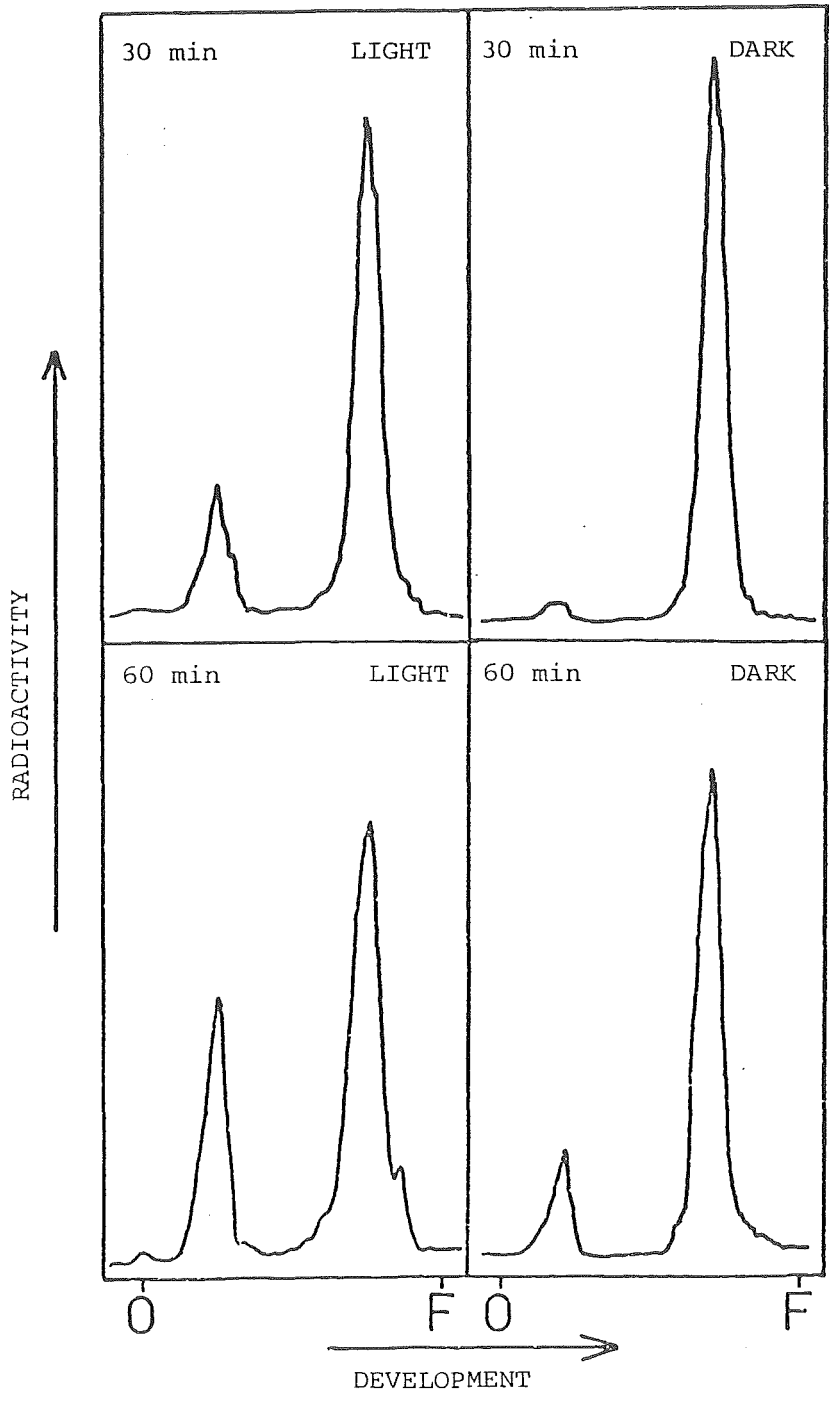

0 min
CONTROL
5 min
LIGHT
5 min
DARK
15 min
LIGHT
15 min
DARK
RADIOACTIVITY
O
F
O
F
DEVELOPMENT

FIG. 3.8 continued.



autoradiogram of one replicate is shown in Fig. 3.9. Six major breakdown products were evident and all increased in amount as the length of the pre-development period was extended. These products were located at the origin, just below the IAA spot (Rf 0.35), at Rf 0.53 - 0.57, and the remaining three were in the Rf 0.67 - 0.84 region.

The proportion of radioactivity in the IAA band and on the remainder of the plate was determined by liquid scintillation analysis (LSA) (section 2.5.7.3). The results are shown in Fig. 3.10. Even when the plates were developed immediately after IAA application, only 93.7% of the radioactivity was recovered as IAA-^{14}C. Pre-incubation of IAA-^{14}C on the plates prior to development, resulted in rapid destruction. A linear relationship between breakdown and length of time on the plate was apparent for 30 min, when more than half the applied IAA-^{14}C had been degraded. The rate of decomposition decreased slightly until 60 min, after which 26.5% of the applied radioactivity remained as IAA-^{14}C.

When 115 Bq IAA-^{14}C was pre-incubated on silica gel plates, 2 or 3 additional metabolites were apparent (Fig. 3.11). The percentage breakdown with time, when IAA-^{14}C was applied at the higher rate (Fig. 3.12), was similar to that observed when 50 Bq IAA-^{14}C was applied. (cf Fig. 3.10).

Further quantification of the decomposition products following 30 min incubation of IAA-^{14}C (above), showed that 68.5% of the breakdown products were located at Rf 0.68 - 0.84 (Table 3.3).

(iii) *Incubation under* N_2 *stream.* TLC plates spotted with 115 Bq IAA-^{14}C were maintained under a constant stream of N_2 prior to development in MEK/hexane. Incubation was for 10 min in normal laboratory lighting and the radioactive compounds on the TLC plate were located using the radiochromatogram scanner (Fig. 3.13). Nitrogen treatment did not prevent IAA decomposition on the plate prior to development.

Figure 3.9: Time-course of breakdown of 50 Bq IAA-^{14}C on silica gel TLC plates in laboratory lighting

IAA-^{14}C was spotted onto a TLC plate at intervals prior to development in IPA. Detection by autoradiography (O = Origin, F = Solvent Front).

Time on plate prior to development.

A	0 min
B	5 min
C	15 min
D	30 min
E	60 min

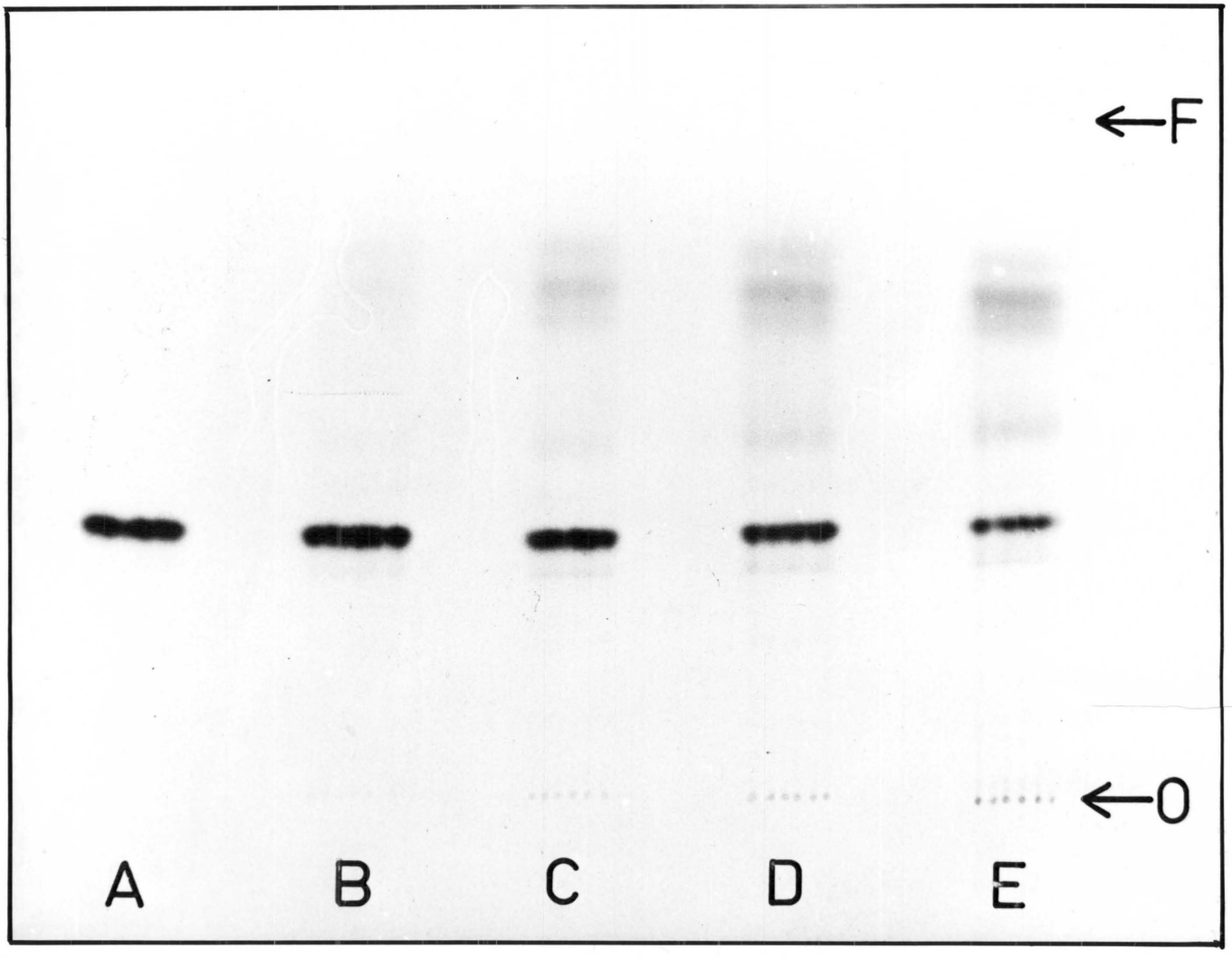

←F
←O
A
B
C
D
E

Figure 3.10: Quantification of IAA-^{14}C breakdown shown in Fig. 3.9

Mean of 2 chromatograms; vertical bars represent twice the standard error of the mean.

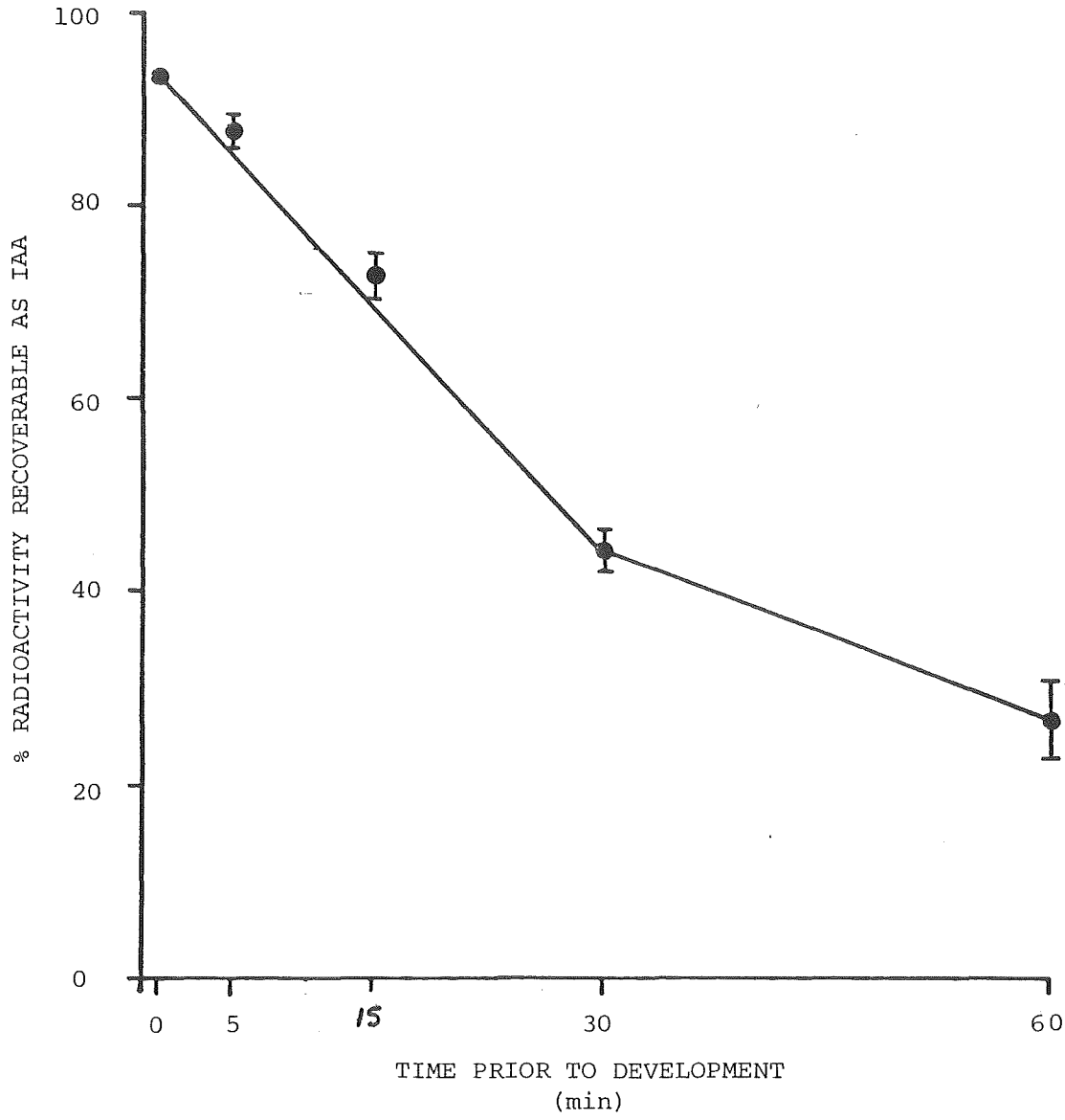

100
80
60
40
20
0
% RADIOACTIVITY RECOVERABLE AS IAA
0
5
15
30
60
TIME PRIOR TO DEVELOPMENT
(min)

Figure 3.11: Time-course of breakdown of 115 Bq IAA-^{14}C on silica gel TLC plates in laboratory lighting

Other details as in Fig. 3.9.

Time on plate prior to development.

A	0 min
B	10 min
C	30 min
D	60 min

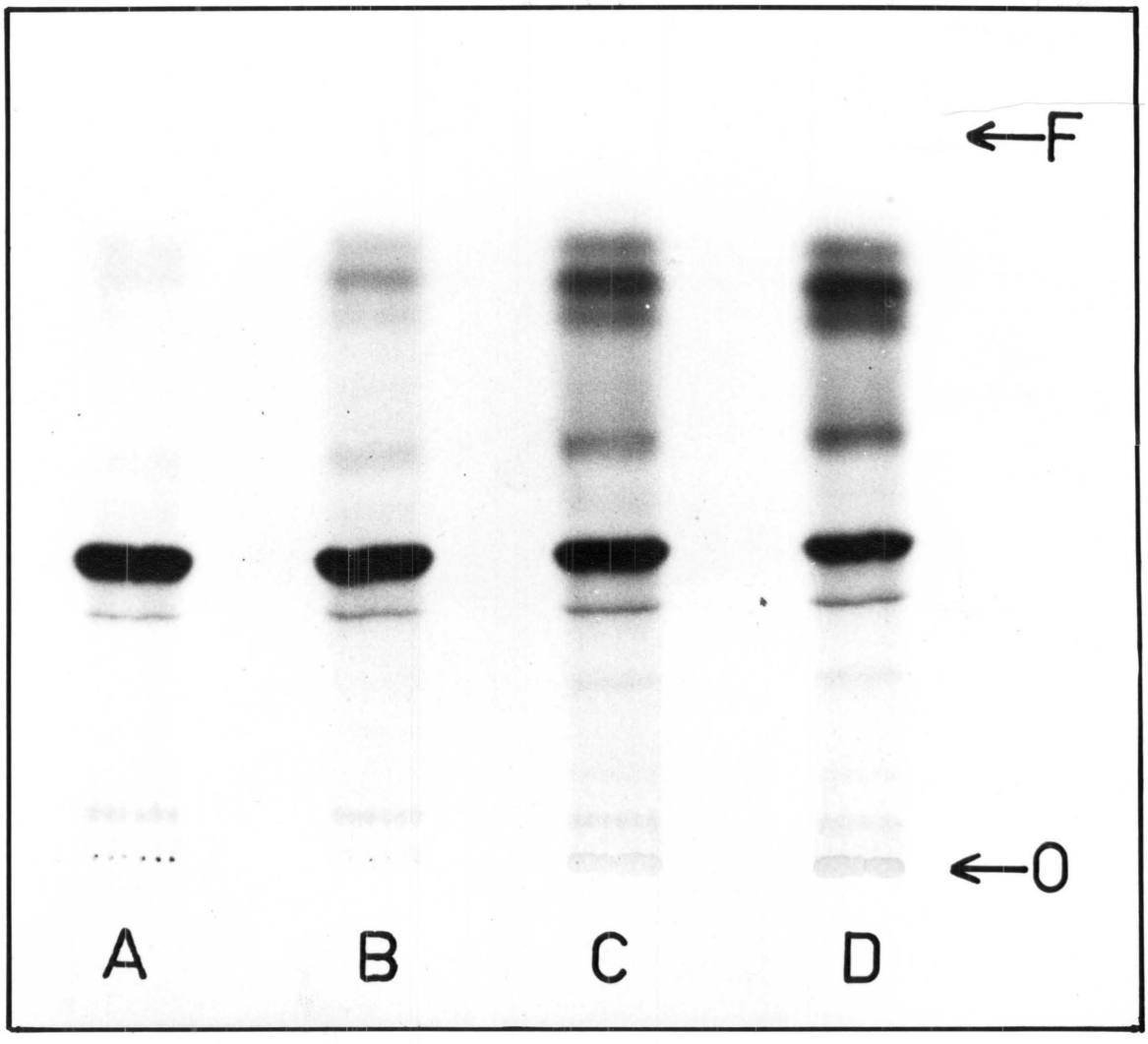

←F
←O
A
B
C
D

Figure 3.12: Quantification of IAA-^{14}C breakdown shown in Fig. 3.11

Mean of 2 chromatograms; vertical bars represent twice the standard error of the mean.

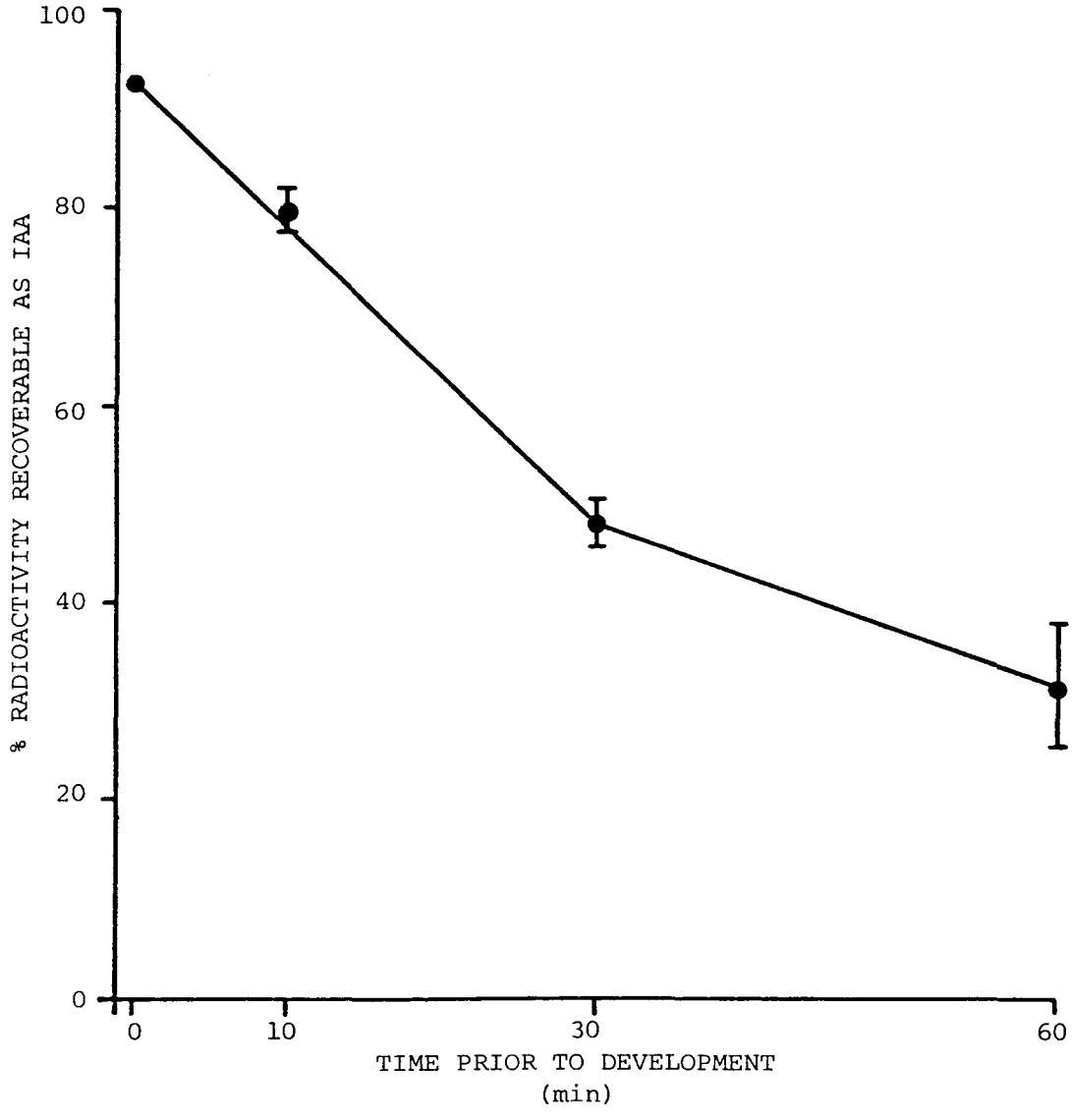

100
80
60
40
20
0
% RADIOACTIVITY RECOVERABLE AS IAA
0
10
30
60
TIME PRIOR TO DEVELOPMENT
(min)

Table 3.3: Distribution of radioactivity on TLC plate incubated 30 min prior to development in iPA

Values are the mean ± S.E. of 2 chromatograms.

Spot	Bq	% of total radioactivity	% of total breakdown product
IAA	58.02 ± 3.85	48.3	
Rf 0.68 - 0.84	42.56 ± 0.50	35.4	68.5
Remainder	19.62 ± 1.38	16.3	31.5
Total	120.20		

A major concern was that after development of the chromatogram, further breakdown of IAA-^{14}C and its products might eventually lead to loss from the chromatogram of the labelled carbon atom. Such an occurrence would be of particular significance when plates were autoradiographed (normally 2-5 weeks) prior to quantification by LSA. Also, any such loss of radioactivity may not occur evenly in the breakdown products and the IAA-^{14}C, and could thus give a false indication of the relative importance of the spots on the chromatogram. To test whether label was being lost from the TLC plate after prolonged periods of incubation, the developed chromatogram showed in Fig. 3.13a was incubated in a drawer at room temperature for 2 weeks before being rescanned. The profile obtained (Fig. 3.13b) was similar to the original scan produced immediately after development of the chromatogram (Fig 3.13a), within the limits of variability of the chromatogram scanner.

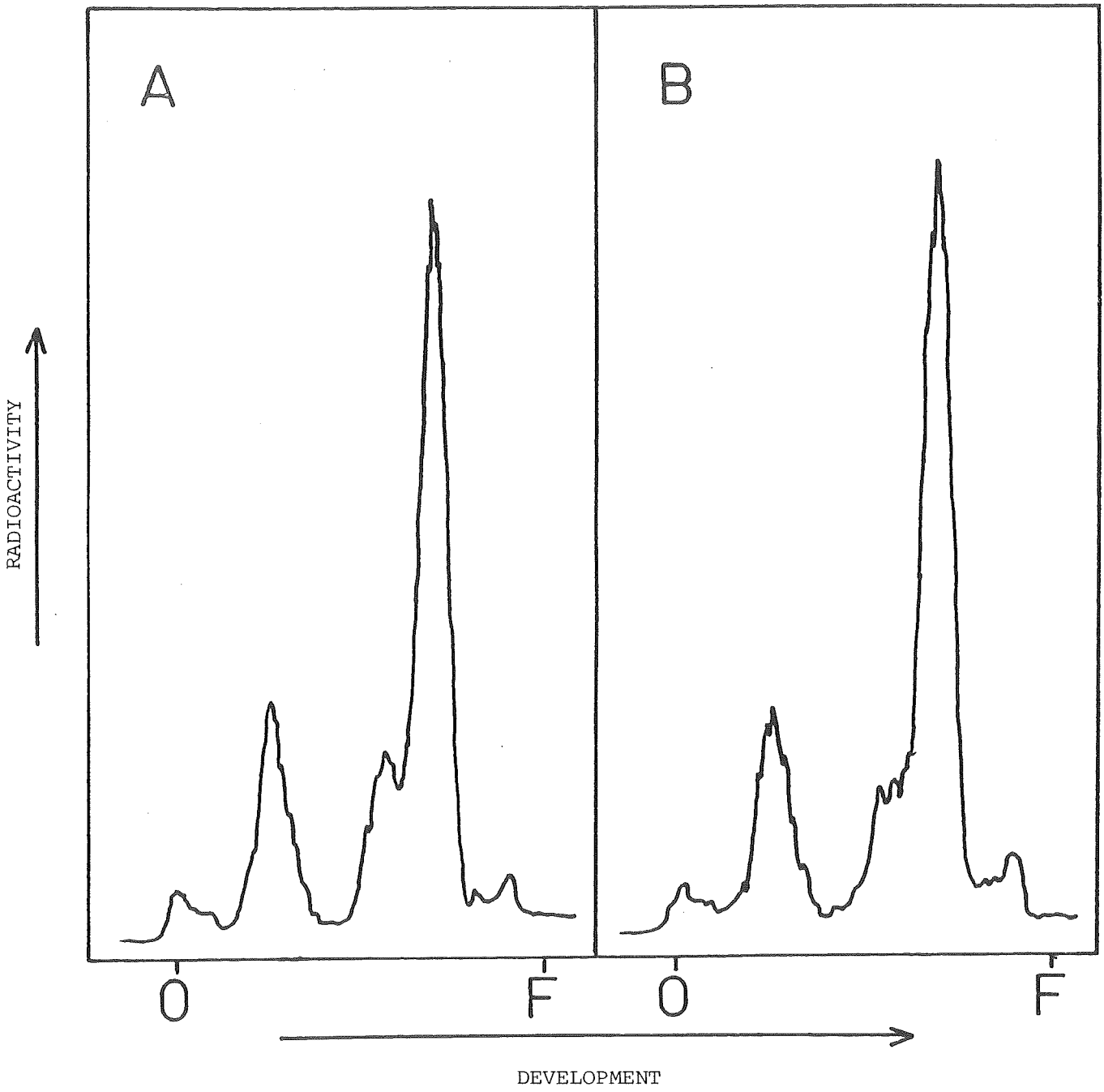
(iv) *Use of unactivated TLC plates.* Muir (1979) reported that heat activation of silica gel plates prior to TLC of the antibiotic, falcarindiol, resulted in breakdown of the compound. However, plates which were not activated

Figure 3.13: Effect of incubation under an N_2 stream on IAA-^{14}C breakdown on silica gel TLC plate

The developing solvent was MEK/hexane. For other details see text.

A. Scanned immediately after development

B. Same plate rescanned after 2 weeks incubation in darkness.


A
B
RADIOACTIVITY
O
F
O
F
DEVELOPMENT

did not cause falcarindiol destruction. In earlier experiments silica gel plates were routinely heat activated prior to use. A comparison was therefore made of IAA-^{14}C destruction on activated and unactivated TLC plates. IAA-^{14}C (160 Bq) was applied to the plates which were incubated in the laboratory for 30 min prior to development in MEK/hexane. Autoradiographs of these chromatograms revealed similar amounts of IAA decomposition on activated and unactivated TLC plates (Fig. 3.14). Activation of the silica gel did result in a slight loss of resolution which was apparent from the more diffuse IAA-^{14}C spot. On the basis of these results a decision was made to use unactivated TLC plates in all further experiments.

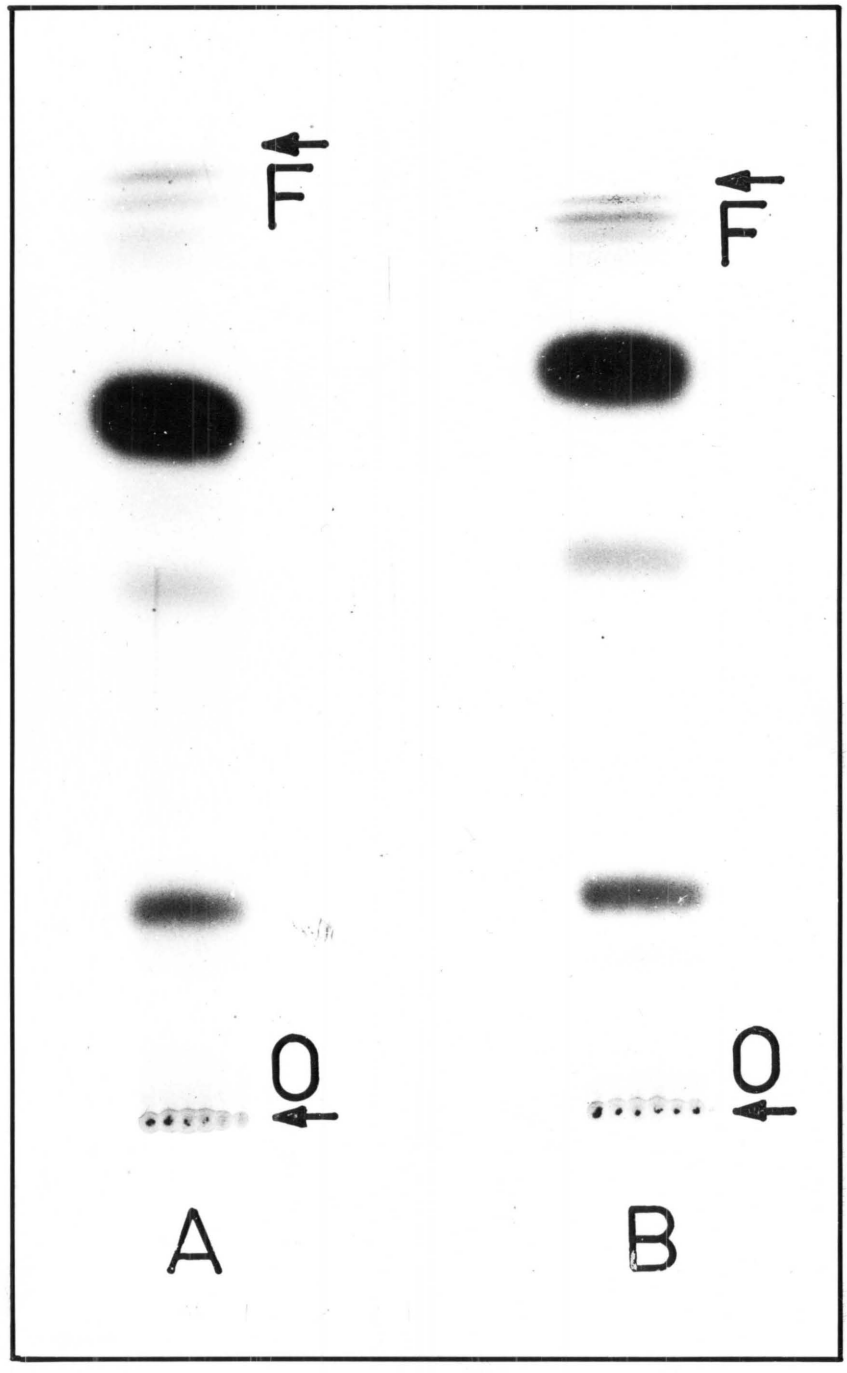
(v) *Visualisation of breakdown products of 'cold' IAA.* TLC plates spotted with 20 µg of IAA (unlabelled) were incubated for 30 min in normal laboratory lighting and developed in MEK/hexane. As an internal standard, 160 Bq IAA-^{14}C was also applied to the origin at the beginning of the experiment. The developed plates were scanned to locate the radioactive metabolites and then sprayed with Salkowski reagent (section 2.5.7.4) to detect indole-containing compounds. IAA produced a strong, vivid pink colour with the Salkowski spray and, in addition, a small amount of brown material was present at the origin. No other Salkowski-positive substances were apparent and there was no colour reaction in the region of the main radioactive breakdown product.

It is possible that insufficient quantities of the breakdown products were present to enable detection with the Salkowski spray. Therefore a 24 h period of IAA incubation on the plate was tried. Again, no Salkowski-positive metabolites were detected, except at the origin. The colour intensity of the IAA spot was much less than when the plates were incubated for only 30 min. Similarly, Ehmann reagent (a mixture of Ehrlich and Salkowski reagents; section 2.5.8.2) did not reveal any IAA breakdown products on the plate.

Figure 3.14: Effect of activation of silica gel TLC plates on IAA-^{14}C breakdown

IAA-^{14}C was incubated on the plate for 30 min prior to development in MEK/hexane. Other details see text.

A. Activated
B. Unactivated


TL
O
A
TL
O
B

(vi) *TLC procedure adopted in this study.* On the basis of the above results relating to IAA breakdown, the following standard procedure was adopted for chromatography of IAA and its derivatives extractable for plant material by 95% ethanol:

- each sample was spotted and developed on 5 x 20 cm TLC plates. This enabled IAA destruction on the plate to be reduced to a minimum, since development could take place immediately after spotting. One disadvantage was that comparison of samples had to be between separate plates rather than on the same TLC plate.

- samples were taken up in minimum methanol to reduce spotting time. Application of the solution to the origin was as a band, approximately 15 mm wide. This operation was carried out under a stream of N_2 in dim light with all natural light excluded.

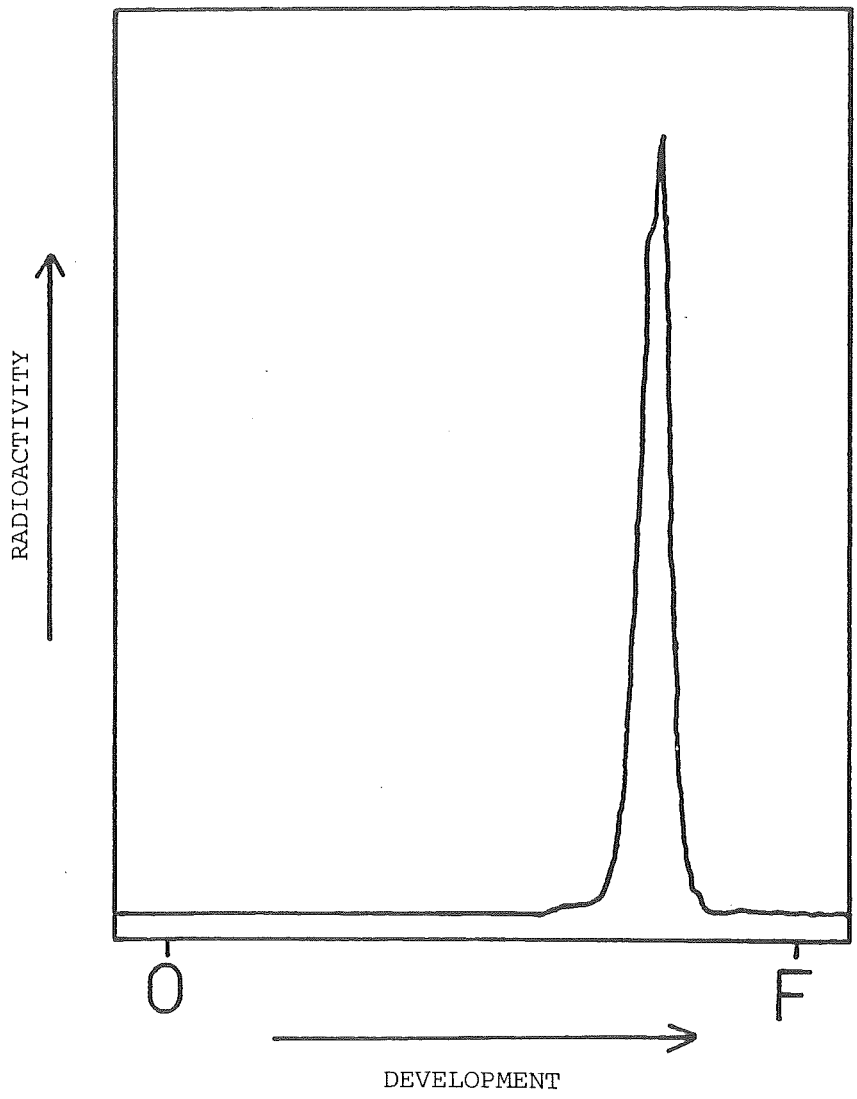
- MEK/hexane was usually employed as the developing solvent. This solvent mixture is very volatile and consequently there was less disturbance to the equilibrium of the chromatography tank when additional new plates were placed in the tank.

3.3.1.2 IAA-2-^{14}C stability during vacuum evaporation

Mann and Jaworski (1970) reported that during rotary evaporation losses of IAA occurred as a result of sublimation. In this investigation, IAA solutions and extracts were concentrated using a test tube evaporator under a vacuum of 30 mm Hg. An experiment was performed to check for IAA loss during this procedure. IAA-^{14}C was dispersed in a solution of 95% aqueous ethanol (v/v) and 10 ml aliquots were delivered into each of ten 18 x 125 mm test tubes. Eight of the test tubes were subjected, in pairs, to varying periods of vacuum evaporation at 35°C, after which the contents of each tube was taken up in 10 ml of 95% ethanol and the radioactivity determined by LSA. As a control, the solution in the remaining 2 test tubes were transferred directly to scintillation vials without test tube evaporation. The results of this experiment

Figure 3.15: TLC of IAA-^{14}C standard (in 95% ethanol) following thin-film evaporation in a test tube

Developing solvent MEK/hexane. Detected by radio-chromatogram scanner.


RADIOACTIVITY
O
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DEVELOPMENT

are shown in Table 3.4. Although there appeared to be a small loss of radioactivity following increasing periods of test tube evaporation (less than 6%, at most), this was not statistically significant (LSD, $P < 0.1$).

Table 3.4: Recovery of radioactivity following varying periods of thin-film evaporation of IAA-^{14}C solutions in a test tube

Treatment	Mean radioactivity (Bq)
Control - no evaporation	124.1 ± 1.11
Taken down to approximately 0.5 ml	123.1 ± 4.00
Almost dry	121.1 ± 3.60
Just dry	119.3 ± 1.78
Given 3 min after liquid disappeared from view	117.1 ± 0.18

In a separate experiment, TLC of IAA-^{14}C standards which had been subjected to test-tube evaporation, revealed that no degradation of the IAA occurred (Fig. 3.15). At all times, IAA-^{14}C standards were chromatographed in parallel with all experimental plant extracts and at no stage, was any decomposition of the standard observed following thin-film evaporation.

3.3.1.3 IAA-^{14}C stability in presence of plant tissue

IAA is destroyed by incubation with 80% ethanolic extracts of leaves, possibly due to peroxidation, catalysed by unsaturated fatty acids (Mann and Jaworski, 1970). To minimise losses of IAA during extraction, these workers maintained the solutions under an N_2 atmosphere and reduced the duration of extraction. In addition the antioxidant,

sodium diethyldithiocarbamate, was added to the extracting solution.

An experiment was carried out to determine whether IAA would break down in the 95% ethanolic (v/v) plant extracts produced in this investigation. Two series of plant extracts were prepared - with and without sodium diethyldithiocarbamate (0.02% w/v), in the extracting solution. Within each series, duplicate vials contained 10 coleoptile tips, 10 mesocotyl segments or 10 grains, taken from 63 h old dark-grown oat seedlings. Absolute ethanol (5 ml) was used as the initial solvent, in an attempt to denature IAA-destroying enzymes that may have been present, and to compensate for the dilution of the alcohol by water in the plant tissue. After 1 h, IAA-^{14}C(967 Bq) was added to each vial and incubated in darkness at 7°C for 12 h. Ten ml of 95% aqueous ethanol (v/v) was then added, and the solution incubated for a further 24 h. Control solutions of IAA-^{14}C without plant tissue were also incubated with each set of extracts.

At the end of the total 36 h incubation period, 10 ml aliquots of solution were taken for LSA. The remainder was concentrated and analysed by TLC in MEK/hexane. From Table 3.5 it can be seen that no loss of radioactivity occurred when IAA-^{14}C was incubated in the presence of plant material, irrespective of whether antioxidant was added to the extracts.

Table 3.5: Recovery of radioactivity after incubation of IAA-^{14}C with plant extracts

For details see text (n = 2).

Treatment	Mean radioactivity (Bq)	
	+antioxidant	-antioxidant
Control (IAA-^{14}C only)	960.0 ± 12.2	970.9 ± 2.6
IAA-^{14}C + coleoptile tips	969.9 ± 10.1	962.2 ± 22.1
IAA-^{14}C + mesocotyl segments	977.9 ± 19.3	978.7 ± 28.2
IAA-^{14}C + grains	961.9 ± 0.5	984.1 ± 15.3

TLC revealed that no decomposition of IAA-^{14}C occurred in the presence of plant tissue, even without antioxidant (Fig. 3.16). Problems were encountered with TLC of solutions containing sodium diethyldithiocarbamate, since crystals formed when the solutions were applied to chromatography plates. This resulted in overloading of the chromatogram and poor resolution of the IAA-^{14}C spot. Because of these difficulties, and the observed lack of IAA-^{14}C destruction in ethanolic solutions without the antioxidant, sodium diethyldithiocarbamate was not used in future experiments.

3.3.2 IAA-^{14}C injection into endosperm

3.3.2.1 Extraction schedule

The transport and metabolism of IAA-^{14}C, following injection into the endosperm of 63 h old dark-grown seedlings, was studied. Experiments were terminated 1, 3, 6 or 24 h after injection and each plant was divided into 10 portions; namely the roots, the grain and 8 shoot segments (see section 2.5.1.2). ^{14}C-labelled compounds were extracted from the plant material using a multiple solvent method (Davies, 1976), which involved successive extraction with 95% ethanol, distilled water, 1N NaOH and finally Soluene (a tissue solubilizer) (Sections 2.5.1.3 and 2.5.2).

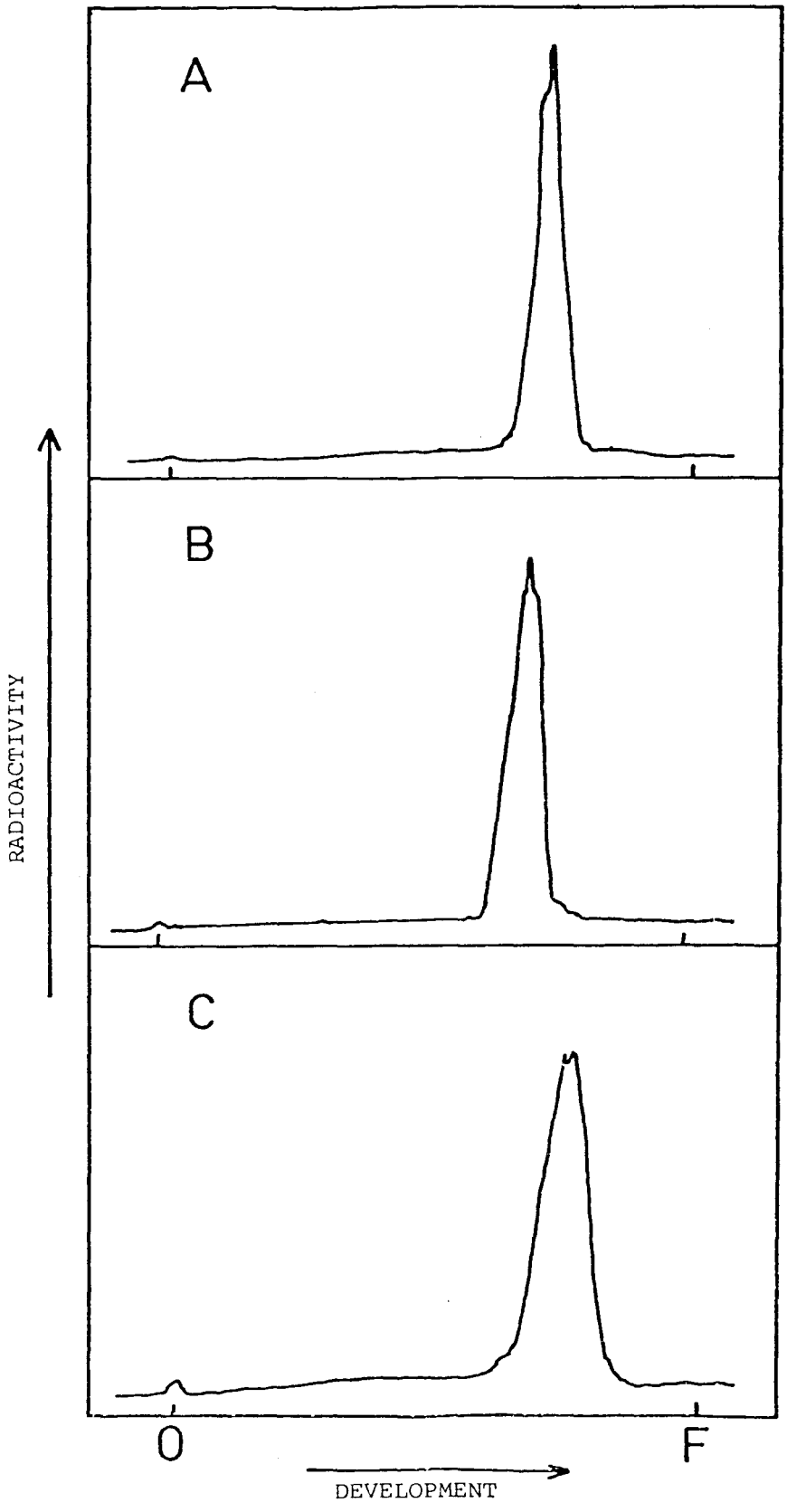
The activity of each solvent fraction was determined by LSA, without prior concentration or purification. Aliquots of the ethanol fraction of the grain, coleoptile tip and upper quarter of the mesocotyl were analysed by TLC (sections 2.5.1 and 2.5.2).

Preliminary experiments revealed that large amounts of radioactivity were extracted from grains by water and NaOH, even after 3 d prior extraction with 95% ethanol. An experiment was performed to determine whether this radioactivity was truly ethanol-insoluble or whether it was merely radioactivity that was difficult to extract. Seedlings were injected with 2 kBq of IAA-2-^{14}C and incubated in darkness

Figure 3.16: TLC with MEK/hexane of IAA-^{14}C following incubation with 95% ethanolic extracts of plant tissue (in absence of antioxidant).

For other details see text

A incubated with coleoptile tips
B incubated with mesocotyl segments
C incubated with grains


A
B
C
RADIOACTIVITY
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F
DEVELOPMENT

for 3 h prior to harvesting. The grains (5) were extracted with 1.5 ml of absolute ethanol for 12 h at 7°C, and then the ethanol decanted and replaced with 1.5 ml 95% aqueous ethanol (v/v). This procedure was repeated 12 hourly, using 95% ethanol, for 4 days. The daily yield of radioactivity was determined by LSA. The grains were then extracted with 3 ml distilled water. The water was changed daily over a 3 d period, after which the tissue was extracted with 2 ml 1 N NaOH at 24°C for 40 h. Finally, the residual radioactivity was extracted by incubation over night in Soluene at 50°C. The radioactivity in each aqueous extract, and in the NaOH and Soluene extracts, was also determined by LSA.

Successive ethanol extractions extracted progressively less radioactivity from the grains (Fig. 3.17), but when the grains were transferred to water, a further significant quantity of activity was extractable. Similarly transfer to NaOH resulted in an additional, large amount of label being released. These results indicated that the material soluble in 95% ethanol, the material soluble in water, and that soluble in NaOH formed distinctly different fractions.

Therefore, for further experiments, use was continued of the standard extraction procedure (sections 2.5.1.3 and 2.5.2), which involved 3d extraction with 95% ethanol, 2d with water, 40 h with NaOH, and overnight in Soluene.

3.3.2.2 Time-course experiments

IAA-2-^{14}C was injected into the grains of 63 h old Avena seedlings, and the transport and metabolism of radioactivity followed over a 24 h period. In separate series of experiments, application of 20 Bq, 200 Bq or 2 kBq IAA-^{14}C were made, representing various degrees of perturbation of the endogenous IAA levels in the grain. Irrespective of the amount injected, the IAA-^{14}C was rapidly metabolised. This is illustrated in Fig 3.18 where 2 kBq of IAA-^{14}C was injected. Within an hour of injection, more than 25% of the IAA-^{14}C had been transformed into 95% ethanol-insoluble forms. After 6h, the amount of water- and NaOH-soluble radioactivity exceeded the amount soluble in 95% ethanol. This situation was not necessarily due to metabolism of IAA since a considerable amount of radioactivity was transported

Figure 3.17: Yield of radioactivity from grains with successive extractions

Grains of 63 h old seedlings, injected with 2 kBq IAA-^{14}C and incubated for 3 h in darkness, were extracted with successive changes of 95% ethanol, water, NaOH and Soluene. For details see text.

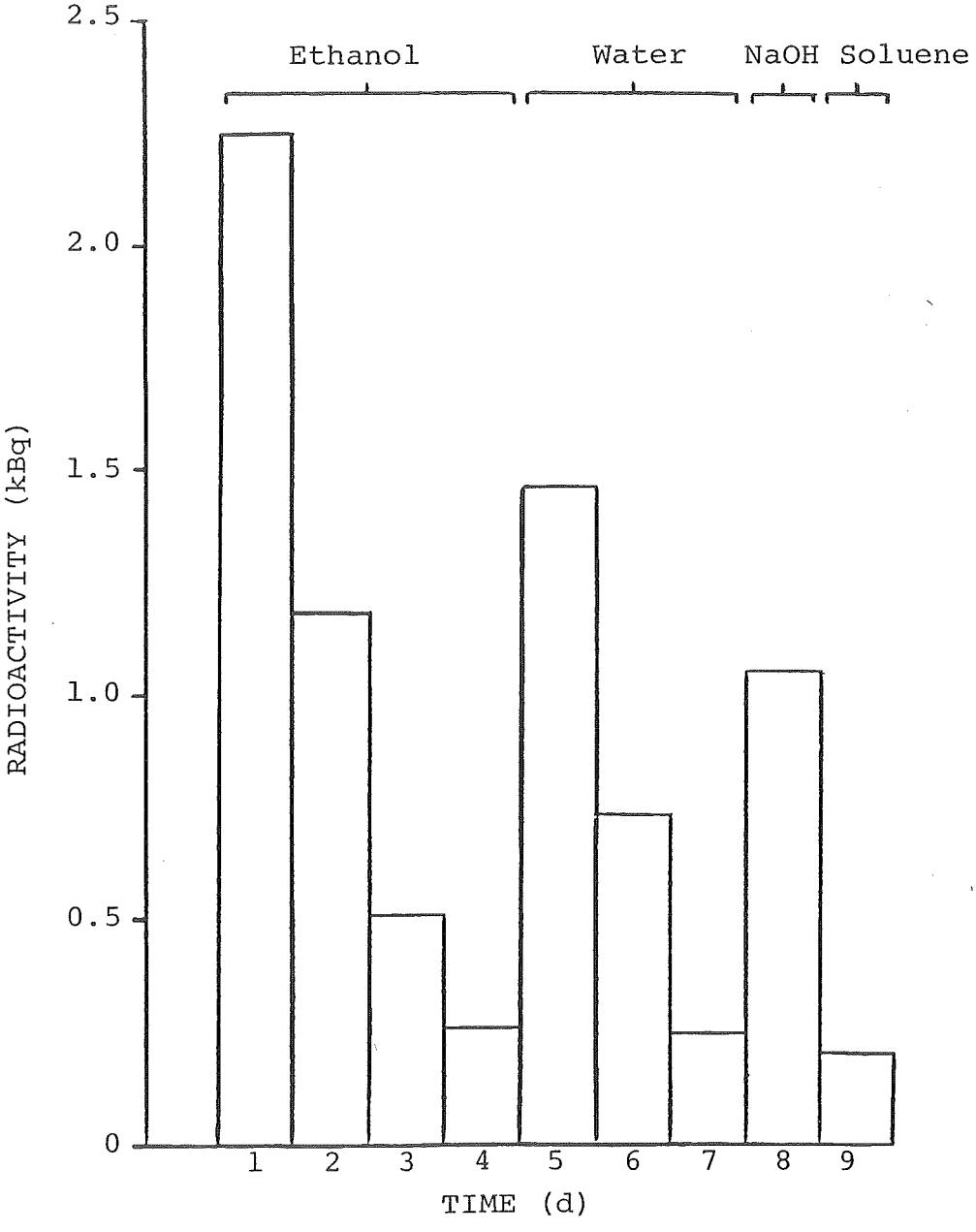
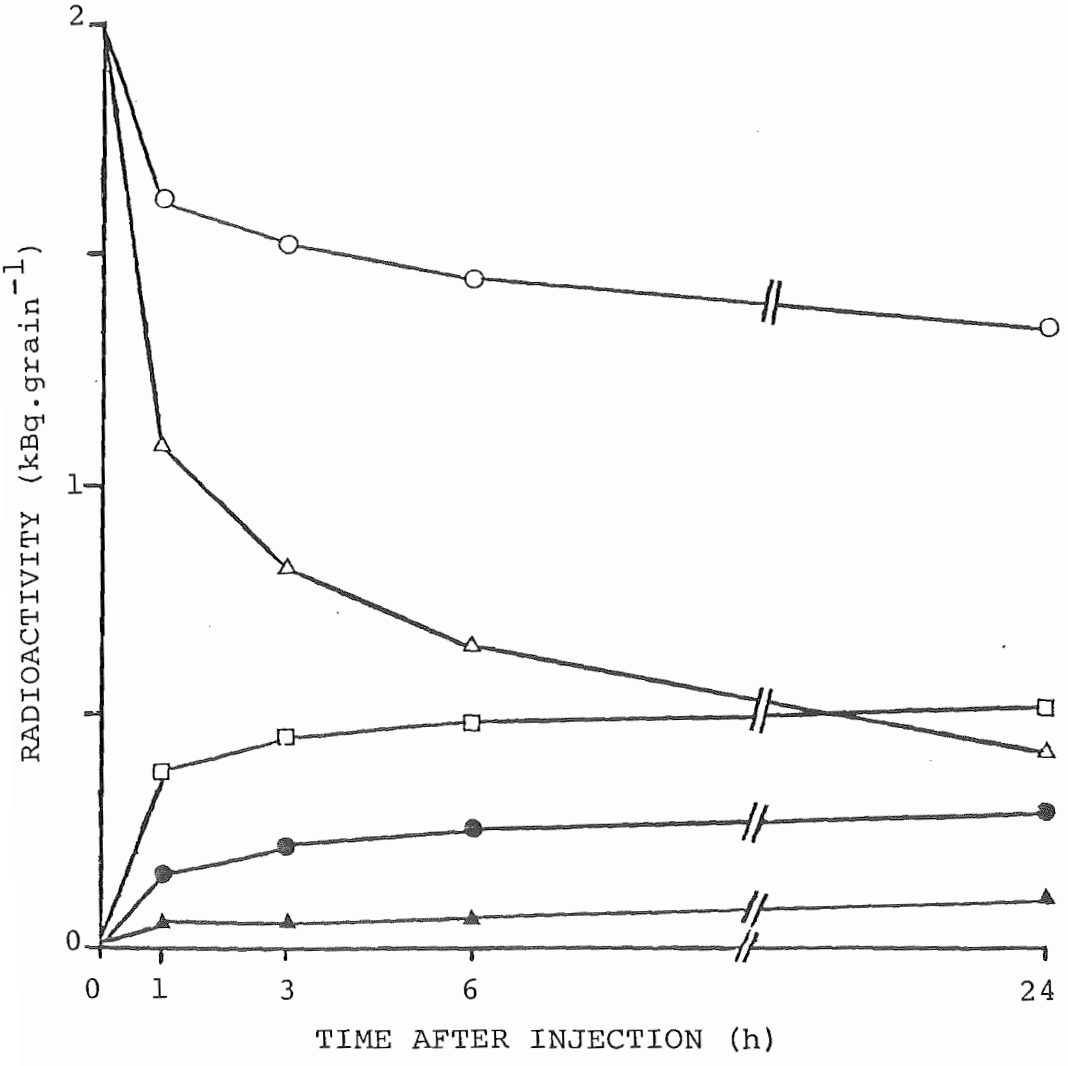

2.5
2.0
1.5
1.0
0.5
0
RADIOACTIVITY (kBq)
Ethanol
Water
NaOH
Soluene
1
2
3
4
5
6
7
8
9
TIME (d)

Figure 3.18: Time-course of extraction of radioactivity from grains by different solvents, following injection of 2 kBq IAA-^{14}C.

○ Total extractable radioactivity
△ 95% ethanol-soluble
□ Water soluble
● NaOH-soluble
▲ Residue (Soluene)


2
1
0
RADIOACTIVITY (kBq.grain^{-1})
0
1
3
6
24
TIME AFTER INJECTION (h)

out of the grain. In fact, after 3 h incubation very little additional water-soluble and NaOH-soluble material accumulated in the grain. The net result, however, was a steady decline in the ratio of ethanol-soluble: water- and NaOH soluble radioactivity.

A more detailed analysis of these results, and those from the 20 and 200 Bq injection experiments, is presented later (section 3.4.1), since their interpretation was influenced considerably by information subsequently gained from studies of the nature of the IAA metabolites.

3.3.3 Analysis of the aqueous extract

The solubility of these radioactive substances suggested that this fraction could consist of bound IAA, possibly in the form of esters (Davies, 1976; Percival and Bandurski, 1976), or associated with peptides (Morris, Briant and Thomson, 1969). Attempts were made to characterize these compounds using water-soluble fractions produced from injections of 2 kBq IAA-^{14}C and incubation times of 6 h.

3.3.3.1 TLC

TLC of the water-soluble fraction, using iPA, failed to move the radioactivity from the origin of the chromatogram, whereas IAA inositols have an Rf of 0.6 - 0.7 in this system (Sheldrake, 1973).

3.3.3.2 Acid hydrolysis

Hydrolysis of the water-soluble material in boiling 1 N HCl for 10 min failed to release any radioactivity that would partition into diethyl ether. Similarly, after direct TLC with iPA, all of the radioactivity continued to remain at the origin.

3.3.3.3 Enzyme treatment

Extracts were treated with a proteolytic enzyme in an attempt to liberate IAA-^{14}C. Water extracts were buffered to give a pH 7.2 0.01 M - phosphate solution. Aliquots (7.0 ml), representing the extract of 10 grains, were

heated with 5 mg Pronase (Sigma) and 4 drops of toluene added as a preservative. After incubation for 16 h at 37°C, the extracts were either chromatographed directly (iPA), or acidified to pH 2.5 with H_2SO_4 and partitioned 3 times against diethyl ether, prior to TLC. In both cases, there was no release of radioactivity after Pronase treatment.

3.3.3.4 Protein determination following column chromatography

Water extract (from 5 grains) was concentrated to 0.1 ml by thin-film evaporation in a test tube and applied to a column (8 x 180 mm) of Sephadex G-25. The sample was eluted from the column with distilled water and 2 ml fractions collected. The elution profile was unaffected by the use of 0.5% KCl (w/v) as the eluant. One quarter of each eluted fraction was assayed by LSA and the remaining 1.5 ml assayed for protein by the Biuret method (section 2.5.8.2).

The radioactivity eluted from the column in a single peak at about V_1 (Fig 3.19). No protein was evident in any of the fractions. The experiment was repeated using 6 times the amount of extract, but again no protein was detectable.

3.3.3.5 Carbohydrate determinations following column chromatography

The aqueous extract was also assayed for carbohydrates, using anthrone reagent (section 2.5.8.2). Sample (0.1 ml), representing an extract of 3 grains, was applied to a column (8 x 180 mm) of Sephadex G-25; 2 ml fractions were collected and 0.5 ml and 1 ml aliquots analysed for radioactivity and carbohydrate respectively (Fig. 3.20a). The radioactivity co-eluted from the column with anthrone-positive material, indicating the presence of carbohydrate. A similar coincidence of radioactivity and carbohydrate was evident when water-soluble, bound-material was chromatographed on a 15 x 200 mm column of Sephadex G-50 (Fig. 3.20b).

Figure 3.19: Elution profile of radioactivity in aqueous extract through a column (8 x 180 mm) of Sephadex G-25

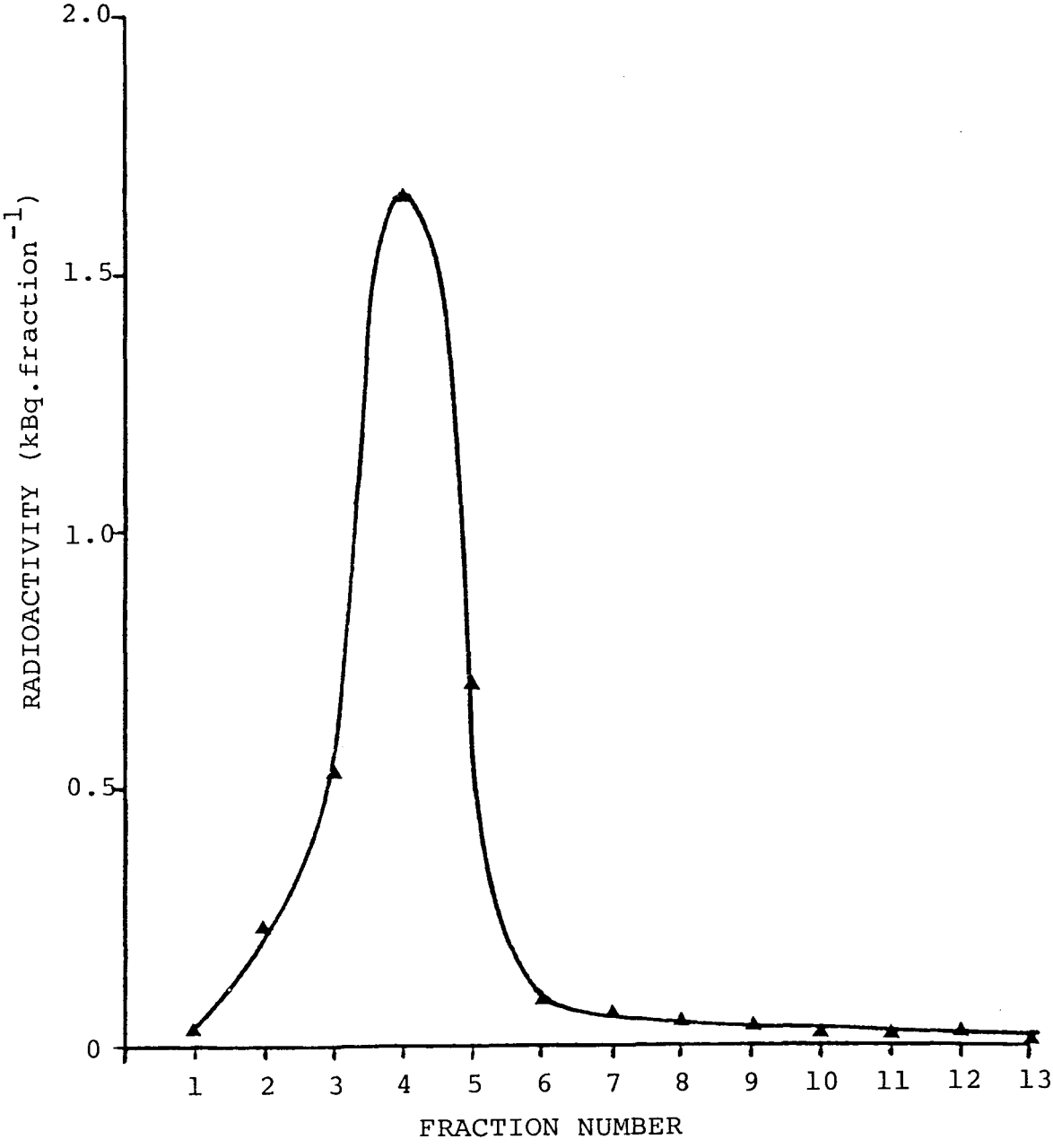
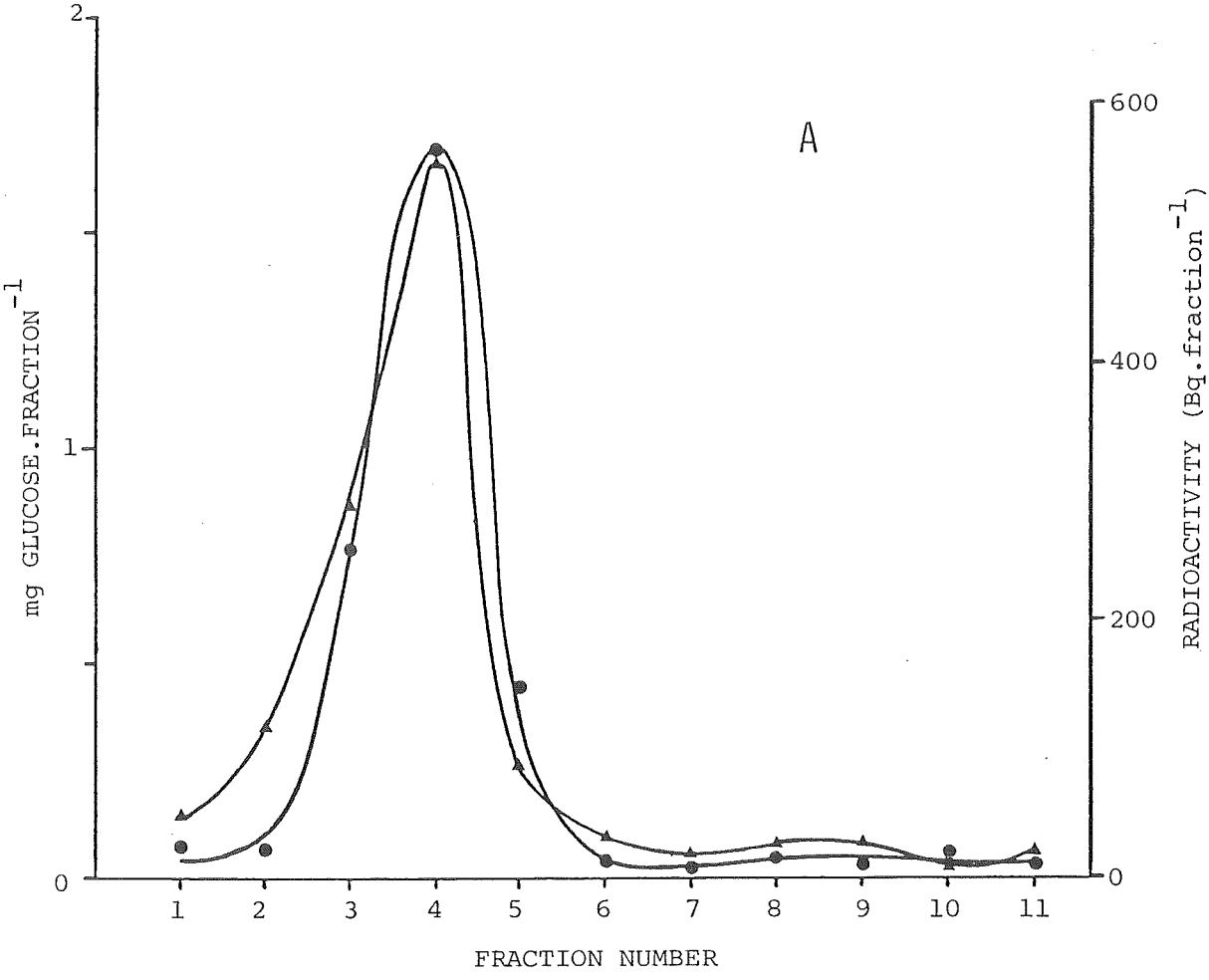
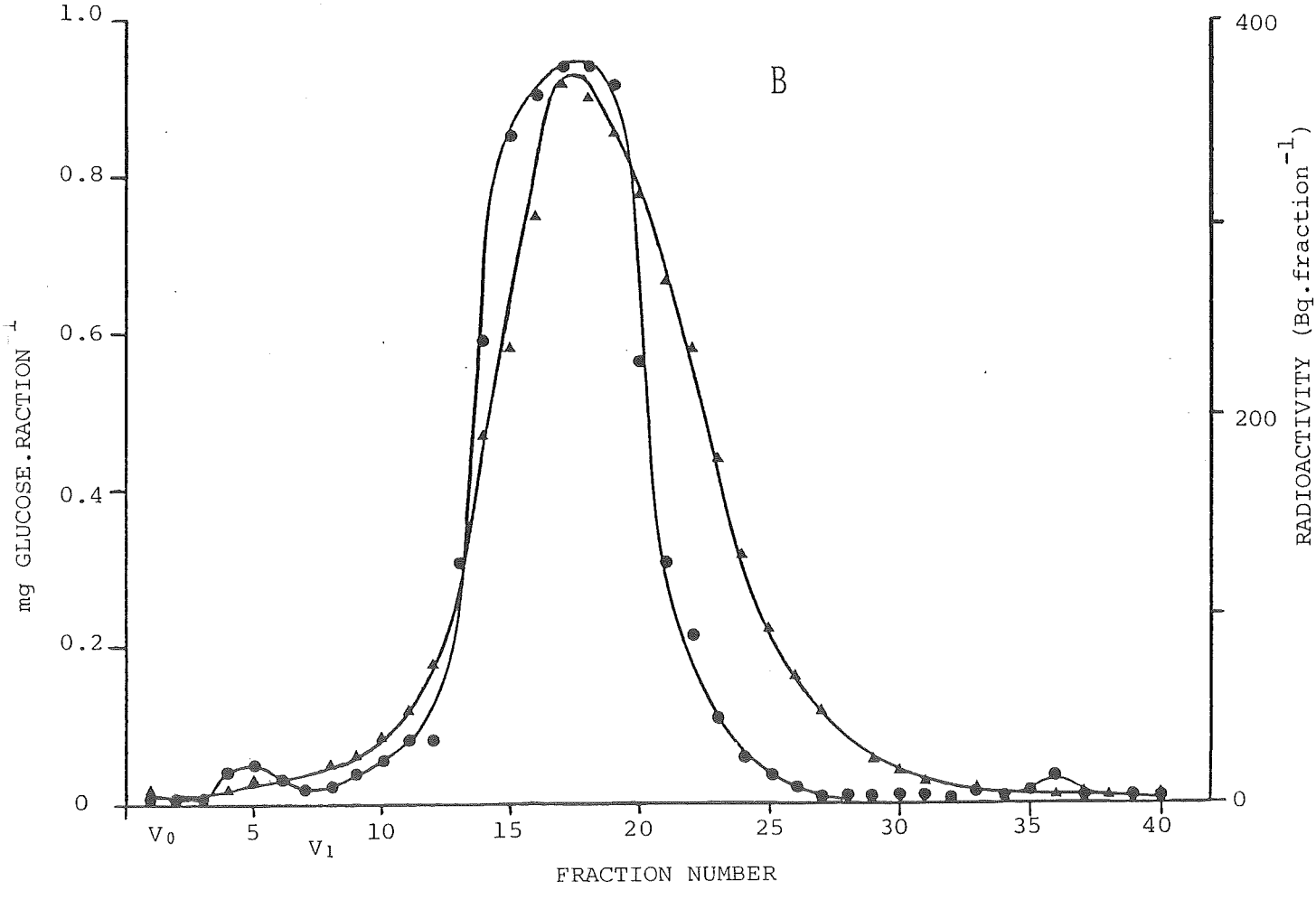

2.0
1.5
1.0
0.5
0
RADIOACTIVITY (kBq.fraction^{-1})
1
2
3
4
5
6
7
8
9
10
11
12
13
FRACTION NUMBER

Figure 3.20: Elution profiles of radioactivity (▲) and carbohydrate (●) in aqueous extract through columns of Sephadex

A Sephadex G-25, 8 x 180 mm column

B Sephadex G-50, 15 x 200 mm column


A
mg GLUCOSE.FRACTION^{-1}
RADIOACTIVITY (Bq.fraction^{-1})
FRACTION NUMBER
0
1
2
200
400
600
1
2
3
4
5
6
7
8
9
10
11


B
mg GLUCOSE.RACTION^{-1}
RADIOACTIVITY (Bq.fraction^{-1})
FRACTION NUMBER
0
0.2
0.4
0.6
0.8
1.0
200
400
V_0
V_1
5
10
15
20
25
30
35
40

3.3.3.6 TLC of hydrolysed extract

Samples of the water-soluble fraction were made 1 N with respect to NaOH, incubated at 24°C for 3 h, then neutralised with 2N HCl. Aliquots were analysed by TLC in iPA. Untreated extract was included for comparative purposes. Radiochromatogram scans of these TLC plates are shown in Fig. 3.21. The bulk of the radioactivity in the untreated extract remained at the origin, but a small amount of activity, with an Rf similar to authentic IAA-^{14}C (0.30 - 0.39), was also present (Fig 3.21a). Following hydrolysis, the activity of the second (IAA) peak increased (Fig. 3.21b).

The mobility of the hydrolysis product on buffered silica gel plates developed with MEK/hexane, was also consistent with the compound being IAA-^{14}C (Fig. 3.22b). The IAA-^{14}C observed in untreated samples developed in iPA (Fig 3.21a), was also present on TLC plates run in MEK/hexane - a neutral solvent system (Fig. 3.22a). This confirmed that the free IAA-^{14}C was not an artefact resulting from hydrolysis of the bound complex during development in the ammoniacal solvent (iPA).

3.3.4 Analysis of aqueous extract from untreated grains

It was considered important to locate and identify the endogenous IAA-esters in Avena grains (Percival and Bandurski, 1976). These could then be used to provide a point of reference for comparison with the ^{14}C-labelled, water-soluble complex.

3.3.4.1 Carbohydrate and indole determinations following column chromatography

Extracts were prepared in the usual manner, but without first injecting the grains with IAA-^{14}C. The extracts were desalted on a column (8 x 150 mm) of Sephadex G-10, concentrated, and applied to a column (8 x 180 mm) of Sephadex G-25. Successive fractions eluting from this column were analysed for the presence of carbohydrates and indole compounds, using the anthrone

Figure 3.21: Radiochromatogram scans of hydrolysed and control aqueous extracts developed in iPA

The position of authentic iAA-^{14}C is indicated.

A. Control
B. Hydrolysed

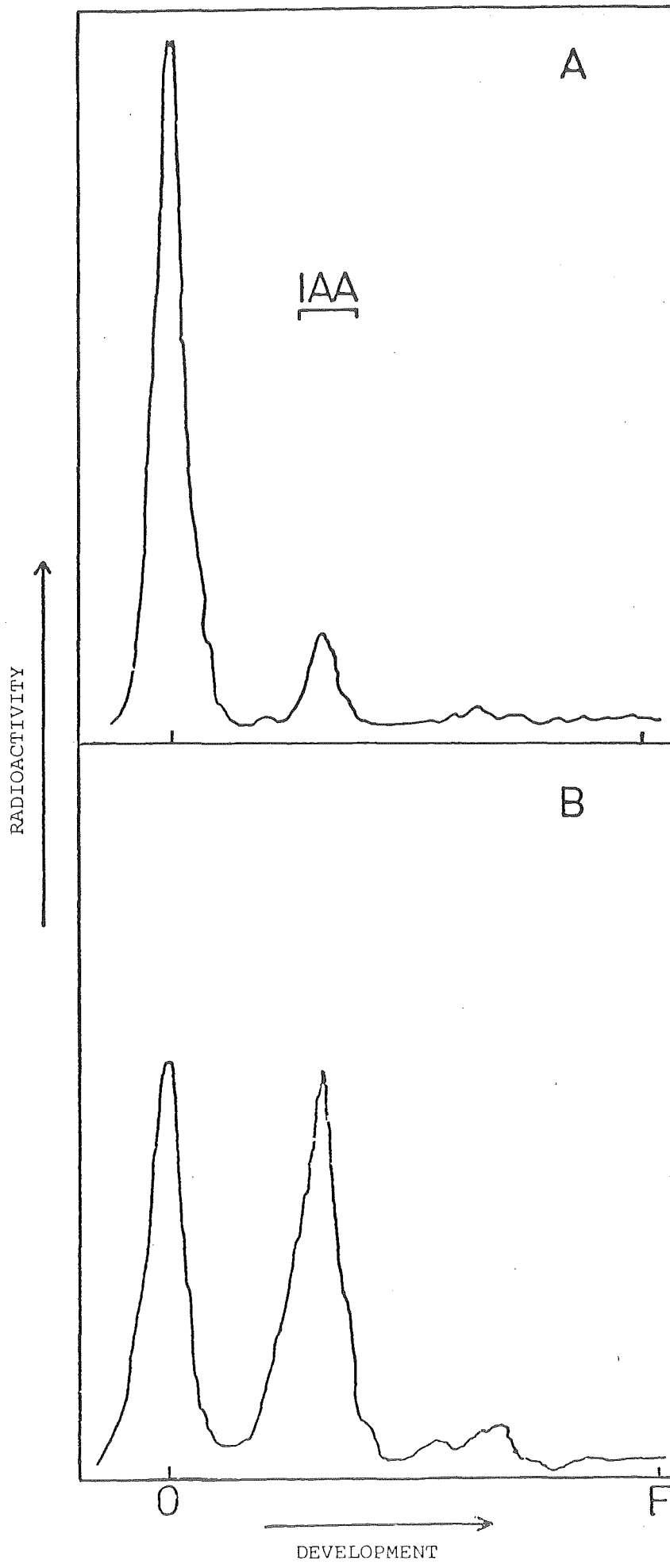
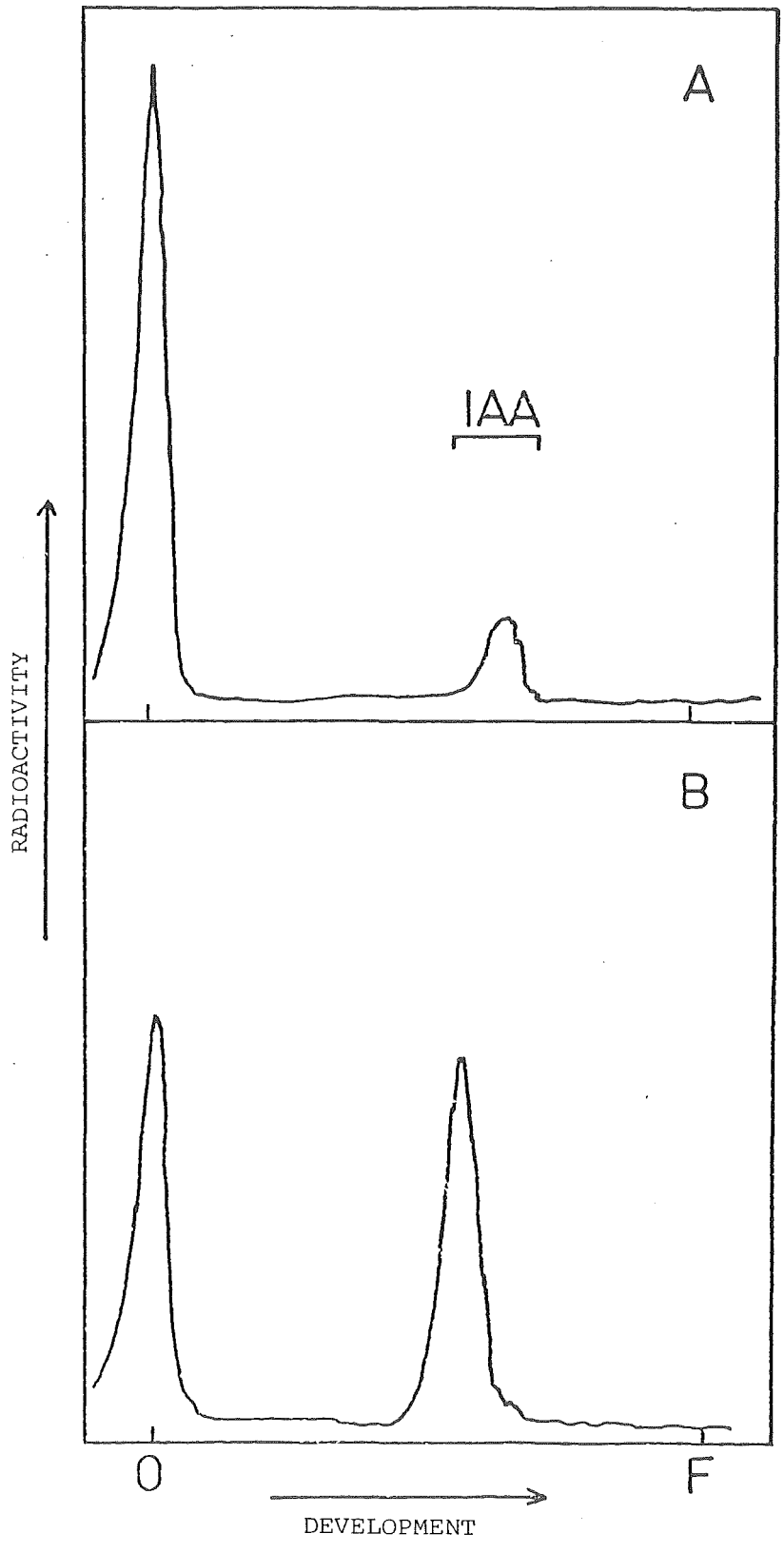

A
IAA
B
RADIOACTIVITY
O
F
DEVELOPMENT

Figure 3.22: Radiochromatogram scans of hydrolysed and control aqueous extracts developed in MEK/hexane

A. Control
B. Hydrolysed


A
IAA
B
RADIOACTIVITY
O
F
DEVELOPMENT

Ehmann tests respectively (section 2.5.8.2) (Fig 3.23a). There was only partial coincidence of Ehmann-positive substances with the carbohydrate peak. The bulk of the indole material was located in fractions 2 and 3, whilst the carbohydrate was concentrated in fraction 4. Resolution of the anthrone-positive and Ehmann-positive peaks was not greatly improved by the use of a larger G-25 column (15 x 300 mm) (Fig 3.23b).

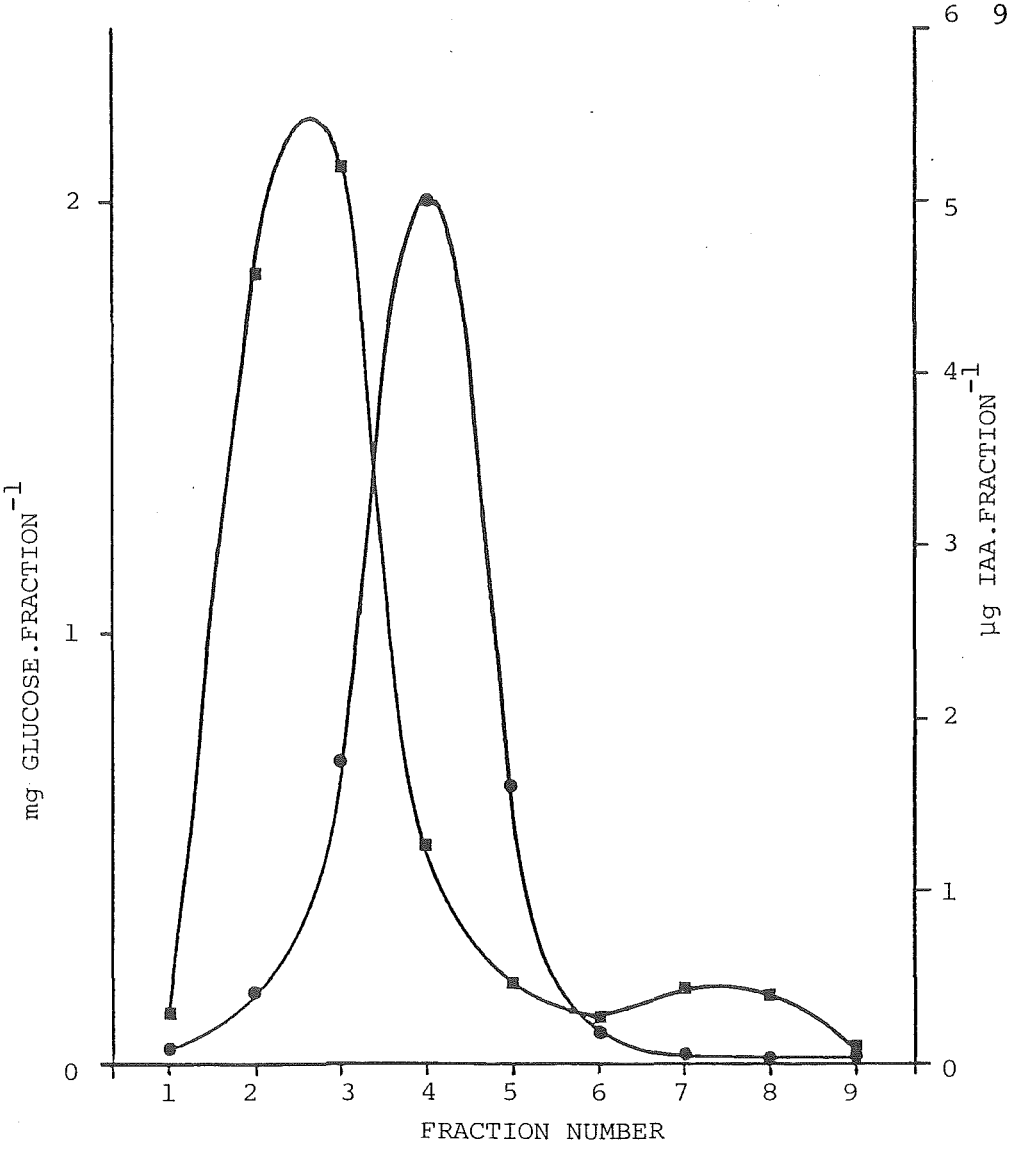
In the above experiments, Ehmann tests were performed directly on the aqueous eluates from the column, whereas Percival and Bandurski (1976) measured IAA released after hydrolysis of the ester complex. Further experiments were performed to determine whether hydrolysis of fractions, prior to indole estimation, would reveal the presence of additional Ehmann-positive compounds. Fractions collected from the G-25 column were hydrolysed in 1N NaOH for 1 h at 25°C, acidified to pH 2.5, and partitioned three times against diethyl ether. The combined ether phases from each fraction were dried and taken up in 10 drops of 50% aqueous ethanol prior to the addition of 0.4 ml of Ehmann reagent, but no Ehmann-positive compounds were detected. Analysis of the aqueous residues revealed that the indole compounds remained in this phase.

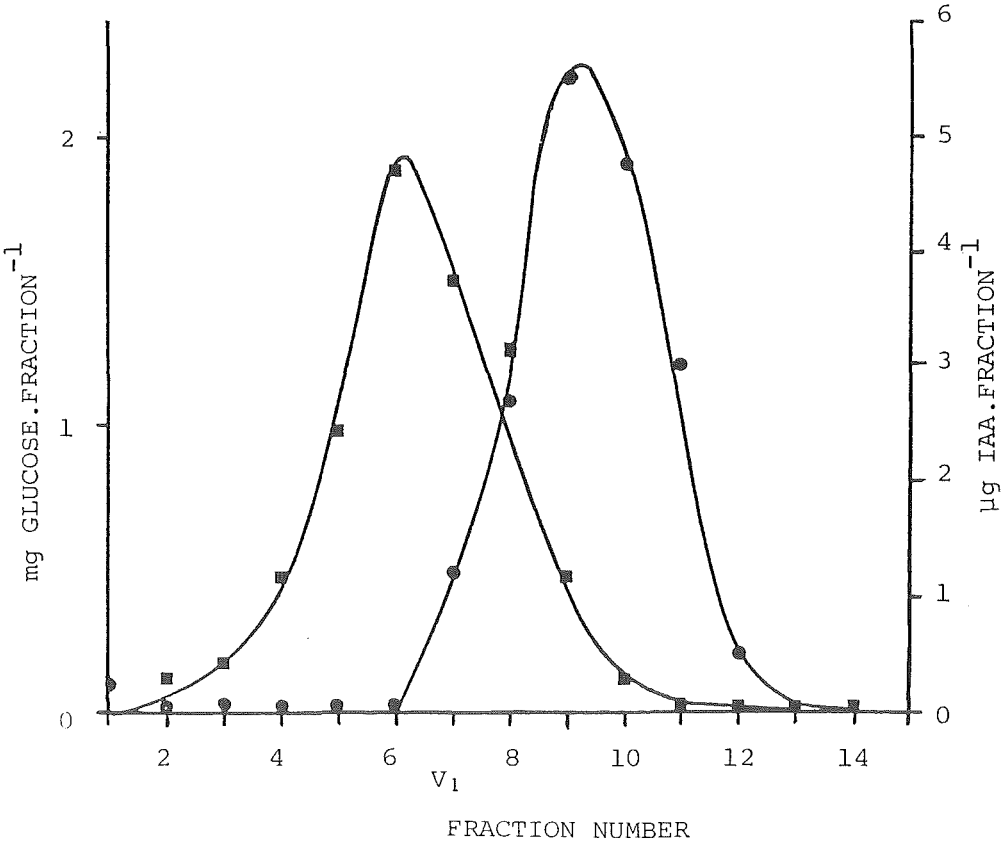
These experiments were repeated twice more, with careful control of the pH of the aqueous extract during ether partitioning, but in each experiment the Ehmann-positive material remained in the aqueous phase. Under the same conditions, authentic IAA in aqueous solution readily moved into the ether phase.

More rigorous hydrolysis was also employed in an attempt to release IAA from the complex. Fractions were made 7 N with respect to NaOH, bubbled with N_2, and sealed in screw cap tubes under a N_2 atmosphere. The tubes were held at 105°C for 4 h, acidified and partitioned against ether. No ether-extractable indole compounds were released by this hot alkaline hydrolysis.

Figure 3.23: Elution profiles of Ehmann-positive (■) and anthrone-positive (●) material in 'cold' aqueous extract of *Avena* grains

A. Sephadex G-25, 8 x 180 mm column
B. Sephadex G-25, 15 x 300 mm column


mg GLUCOSE.FRACTION^{-1}
µg IAA.FRACTION^{-1}
FRACTION NUMBER
0
1
2
3
4
5
6
7
8
9


mg GLUCOSE.FRACTION^{-1}
µg IAA.FRACTION^{-1}
V_1
FRACTION NUMBER
0
2
4
6
8
10
12
14

3.3.4.2 Protein determinations

UV monitoring of the eluate of the Sephadex column revealed the presence of an absorbance peak at 280 nm in both the anthrone-positive and Ehmann-positive fractions. The latter was to be expected, because indole has a characteristic absorbance at 279 nm. However, since carbohydrates do not show strong UV absorption, it was considered that the UV absorbance of the anthrone-positive fractions was probably due to the presence of other contaminating or associated substances. It seemed unlikely that these substances were phenolics, since the phenolic material in Percival and Bandurski's (1976) ester, absorbed at 320 nm. However, proteins are characterized by absorbance peaks at 254 nm and 280 nm, so further tests for the presence of proteins were carried out.

The original protein determinations used the Biuret reagent and, although this test is relatively insensitive, it is more specific for peptide bonds and was preferred to the Folin-Lowry method which reacts with indole and other groups.

A simple qualitative but sensitive, ninhydrin test was therefore employed to indicate the presence of amino groups (section 2.5.8.2). Ninhydrin has also been used to detect indole compounds (Stahl, 1969), but no reaction was obtained from IAA solutions ranging in concentration from 0.5 µg to 20 µg ml^{-1}.

These tests revealed the presence of ninhydrin-positive substances which exactly co-eluted from the column with the anthrone-positive material. This co-elution was observed with both the small and large G-25 columns and the intensity of the ninhydrin colour appeared to parallel the amount of carbohydrate in each fraction. The ninhydrin-positive substances were not associated with the Ehmann-positive material.

3.3.5 Further investigation of the water-soluble material

3.3.5.1 Carbohydrate, protein and indole determinations on radioactive aqueous extract

Extracts of grains which had not been injected with IAA-^{14}C were analysed for the presence of ninhydrin-positive and Ehmann-positive compounds, in addition to the usual

tests for carbohydrate and radioactivity, following elution from a column (15 x 300 mm) of Sephadex G-25. The results are shown in Fig 3.24. As with extracts from untreated grains, Ehmann-positive material was present, most of which eluted ahead of the carbohydrate peak. Exact co-elution of carbohydrate, ninhydrin-positive material and radioactivity was observed.

The co-elution of ninhydrin-positive, anthrone-positive and radioactive material, and the fact that IAA-^{14}C could be released by alkaline hydrolysis, gave the complex some of the characteristics of the IAA-glucoprotein ester descrobed by Percival and Bandurski (1976). However, the rigorous hydrolysis needed to liberate the IAA-^{14}C, and the apparent lack of associated indole material, was not consistent with the view that the complex was an IAA-ester.

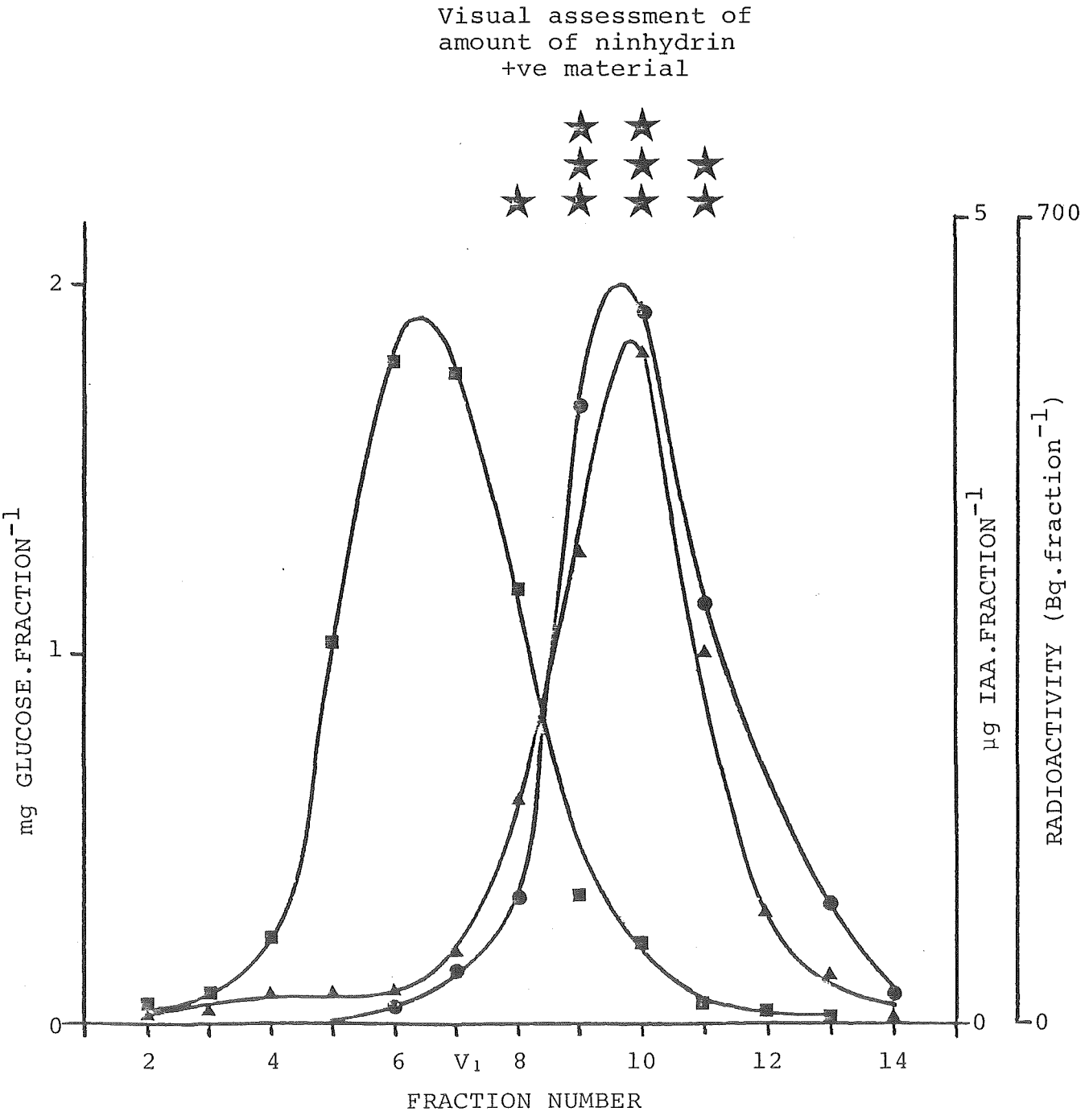
3.3.5.2 Preparation of 'crude ester' fraction from 50% acetone extract of untreated grains

According to the literature, the endogenous IAA-ester would be expected to be insoluble in 95% ethanol, but soluble in water (Davies, 1976). One possibility was that ethanol extraction caused denaturation of the protein component of the complex, thereby rendering the whole molecule insoluble in water.

Intact grains from 63 h old, untreated seedlings were extracted in 50% aqueous acetone (w/v) and the IAA-ester fraction obtained by acetone precipitation, using a method similar to that of Percival and Bandurski (1976) (section 2.5.5). This 'crude ester' fraction was desalted on Sephadex G-10, concentrated and subjected to gel filtration on Sephadex G-25. Analysis of the fractions eluted from the column, revealed that the 'ester' extracted by 50% acetone was identical to the material present in the water-soluble extract. Co-elution of anthrone-positive and ninhydrin-positive material was observed and, as with the water fraction (section 3.3.5.1), Ehmann-positive substances were eluted ahead of the carbohydrate and protein.

Similar extracts were produced from grains which had been injected with IAA-^{14}C. The radioactivity co-chromatographed with ninhydrin- and anthrone-positive material on a

Figure 3.24: Elution profiles of Ehmann-positive (■), anthrone-positive (●), and ninhydrin-positive substances (★), and radioactivity (▲) from aqueous extract through a column (15 x 300 mm) of Sephadex G-25


Visual assessment of
amount of ninhydrin
+ve material
mg GLUCOSE.FRACTION^{-1}
µg IAA.FRACTION^{-1}
RADIOACTIVITY (Bq.fraction^{-1})
FRACTION NUMBER
V_1
0
1
2
5
700
2
4
6
8
10
12
14

column of Sephadex G-25 and in every way appeared identical to the radioactive water-soluble bound complex.

3.3.5.3 Preparation of extracts from macerated grains

It remained a matter of concern that the IAA esters described by Percival and Bandurski (1976) were not apparent in any of the extracts prepared thus far, especially since they were absent from the 'crude ester' fraction prepared by precipitation of 50% acetone extracts. It was possible that the esters were altered during extraction, since whole grains were used in these experiments, whereas Percival and Bandurski's extracts were prepared from ground endosperms (i.e. 'flour'). The slower penetration of solvent into the intact endosperms could have resulted in artefacts, particularly if enzyme inactivation was significantly delayed. To test this hypothesis, injected and control grains were finely chopped prior to extraction in 50% acetone, and the 'crude ester' fractions obtained by precipitation of the acetone extracts. These extracts had identical elution profiles to the water-soluble bound complex when chromatographed on columns of sephadex G-25, and exhibited similar reactions with ninhydrin, anthrone, and Ehmann reagent.

3.3.5.4 TLC of labelled water-soluble material from intact grains and labelled 'crude ester' from intact and macerated grains

UV spectrophotometric analysis of the fractions eluting from the Sephadex G-25 column, suggested that each fraction was not homogeneous, but contained a number of compounds; and that the resolution of the column was inadequate to separate what appeared to be two distinct peaks - the Ehmann-positive material and the labelled complex. Further analysis of the Sephadex-purified radioactive extracts was undertaken using TLC on silica gel.

The labelled fractions collected from the Sephadex column were combined, concentrated and streaked on duplicate TLC plates (20 x 20 cm) and developed with either chloroform/methanol/water ($CHCl_3$/MeOH/H_2O) or ethyl acetate/ethanol/formic

acid/water (EtOAc/EtOH/HCOOH/H_2O) (section 2.5.7.1). Normally two identical chromatograms were developed in each solvent. One was tested for amino groups using ninhydrin, the other was tested for carbohydrates with diphenylamine/aniline reagent (section 2.5.7.4). Following colour development, both plates were subjected to autoradiography.

Problems were encountered with the preparation of the sugar visualising reagent. On addition of the orthophosphoric acid, precipitation of the aniline occurred. To overcome this, the acid was applied to the plate as a separate spray, following application of the diphenylamine/aniline reagent. Subsequently it was found that the acid spray could be omitted without affecting visualisation of the sugar spots. Because residual orthophosphoric acid caused damage to the X-ray film during autoradiography, the modified method with the omission of the acid spray was adopted as standard procedure.

Another problem occurred when plates sprayed for carbohydrate and amino acids were heated together. Areas that had been sprayed with ninhydrin developed a strong purple background colouration. No such background occurred in the absence of the sugar reagent. It was presumed that fumes from the heated aniline which contains an amino group, reacted with the ninhydrin to produce the background colour. This problem was easily avoided if the TLC plates were kept separate during heating, or in the case where 2 halves of a single chromatogram were used for each analysis, if the ninhydrin-positive spots were recorded prior to visualisation with the sugar reagent.

Aliquots of the purified water-soluble bound complex from intact grains, and the acetone-precipitated bound complex from both intact and macerated grains, were chromatographed in $CHCl_3$/MeOH/H_2O and analysed for the presence of radioactivity and amino groups (Fig. 3.25), as well as radioactivity and sugars (Fig. 3.26). Aliquots of the same extracts were also chromatographed in EtOAc/EtOH/HCOOH/H_2O (Figs 3.27 and 3.28).

The chromatographic behaviour, and coincidence between radioactivity, sugar and amino groups was identical in all 3

Figure 3.25: TLC in $CHCl_3/MeOH/H_2O$ of radioactive aqueous extract from intact grains, and acetone-precipitated material from intact and macerated grains - radioactivity and amino group analysis.

(O = Origin, F = Solvent Front)

H. Acetone-precipitate, intact grains
I. Aqueous extract, intact grains
J. Acetone-precipitate, macerated grains

AMINO GROUPS

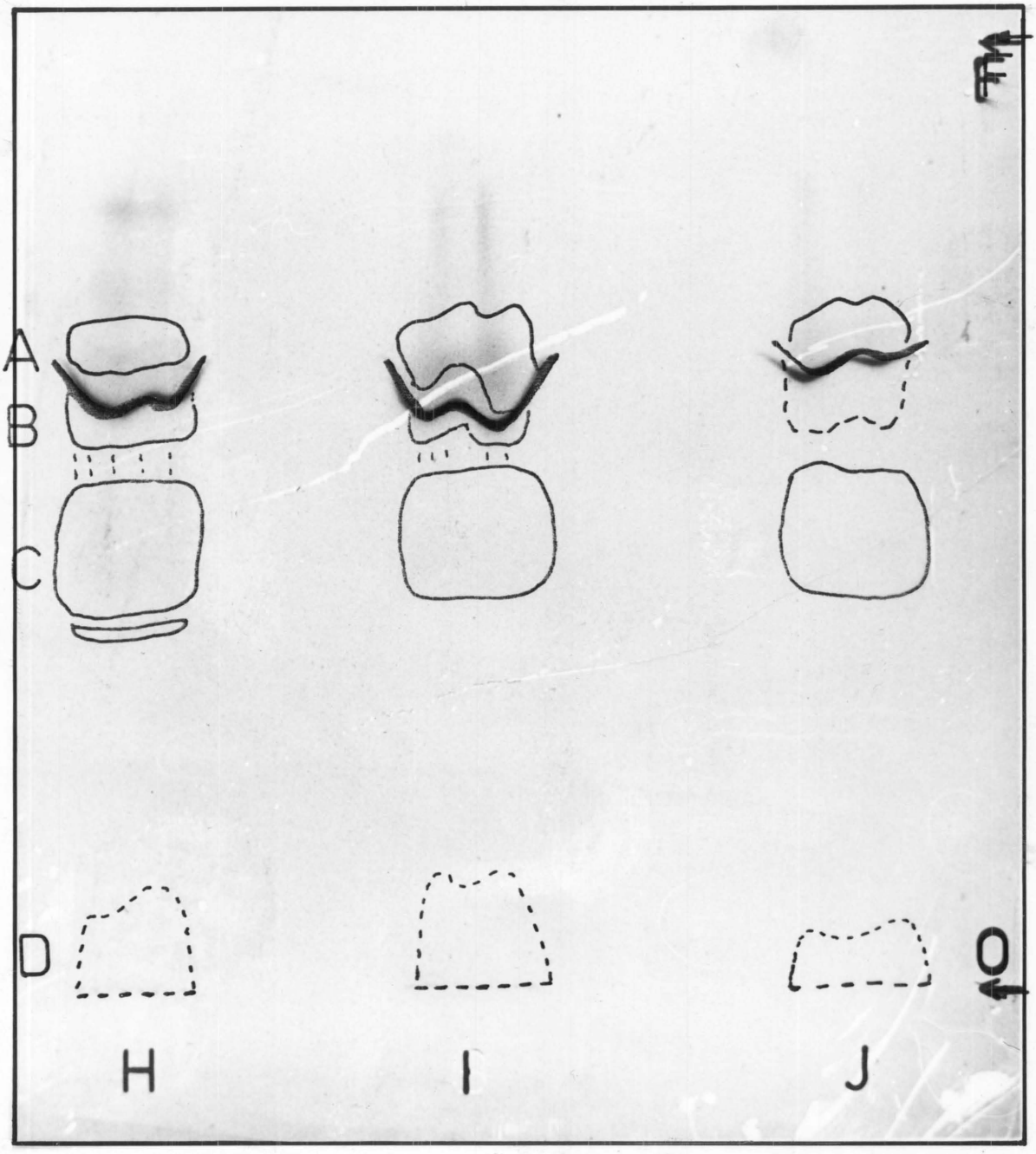
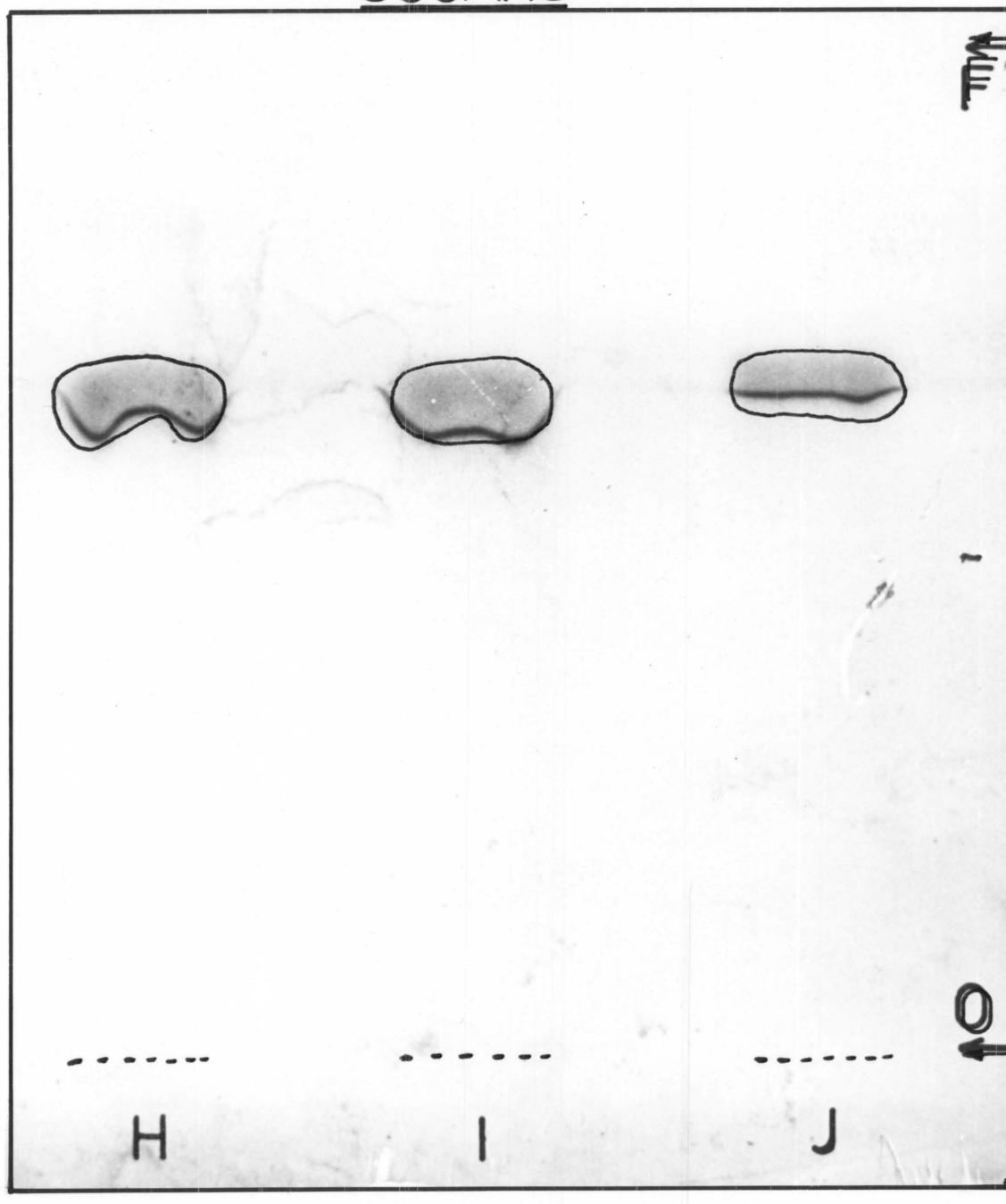


Figure 3.26: Legend as in Fig. 3.25 except analysed for radioactivity and sugars

SUGARS



extracts. This was the case in both TLC solvent systems. The slight differences in mobility between the samples, particularly in $CHCl_3/MeOH/H_2O$ (Figs 3.25 and 3.26), was considered to be a function of the level of sample loading, signified by the distinctive 'W' shape when application rates were high. Mobilities in $EtOAc/EtOH/HCOOH/H_2O$ were more similar (Figs. 3.27 and 3.28). Because of the potential overloading problem, care was taken in future experiments to keep application rates of samples uniform (as monitored by intensity of spray reactions and radioactivity). If applications were too low, detection of the carbohydrate material was difficult.

In $CHCl_3/MeOH/H_2O$ the bulk of the radioactivity was located in a single spot, Rf 0.61 - 0.70, which co-chromatographed with material giving a positive sugar reaction (Fig. 3.26). This spot fluoresced blue-white under UV light, charred on heating with conc H_2SO_4 and gave a green colour reaction when sprayed with 0.2% anthrone in conc H_2SO_4. On the trailing edge of this spot was a concentrated band of radioactivity, which, when sample loading was high, formed a 'W' shape. This band was also strongly ninhydrin-positive (purple) (Fig. 3.25). Four other main areas of ninhydrin-reactive material were present on the chromatogram; labelled A (orange), B,C and D (purple) in Fig. 3.25 . Only compound D was associated with substances that reacted with the sugar reagent and were radioactive, and then there was only partial co-chromatography.

Two other bands of radioactivity were evident at Rf 0.81-0.86 and Rf 0.75-0.79 (Fig 3.25 and 3.26). Some 'tailing' was evident between these two spots, and between the lower spot and the major radioactive spot. No substances which reacted with either ninhydrin or the sugar reagent were apparent in the region of the two minor radioactive spots.

Two main radioactive spots were present on TLC plates developed with $EtOAc/EtOH/HCOOH/H_2O$; the stronger one at Rf 0.70 - 0.77, the weaker at Rf 0.85 - 0.89 (Figs. 3.27 and 3.28). A dark, ill-defined band was apparent on the autoradiograph, about Rf 0.66-0.69. Since the band extended

Figure 3.27: TLC in EtOAc/EtOH/HCOOH/H_2O of radioactive aqueous extract from intact grains, and acetone-precipitated material from intact and macerated grains - radioactivity and amino group analysis.

(O = Origin, F = Solvent Front)

H. Acetone-precipitate, intact grains
I. Aqueous extract, intact grains
J. Acetone-precipitate, macerated grains

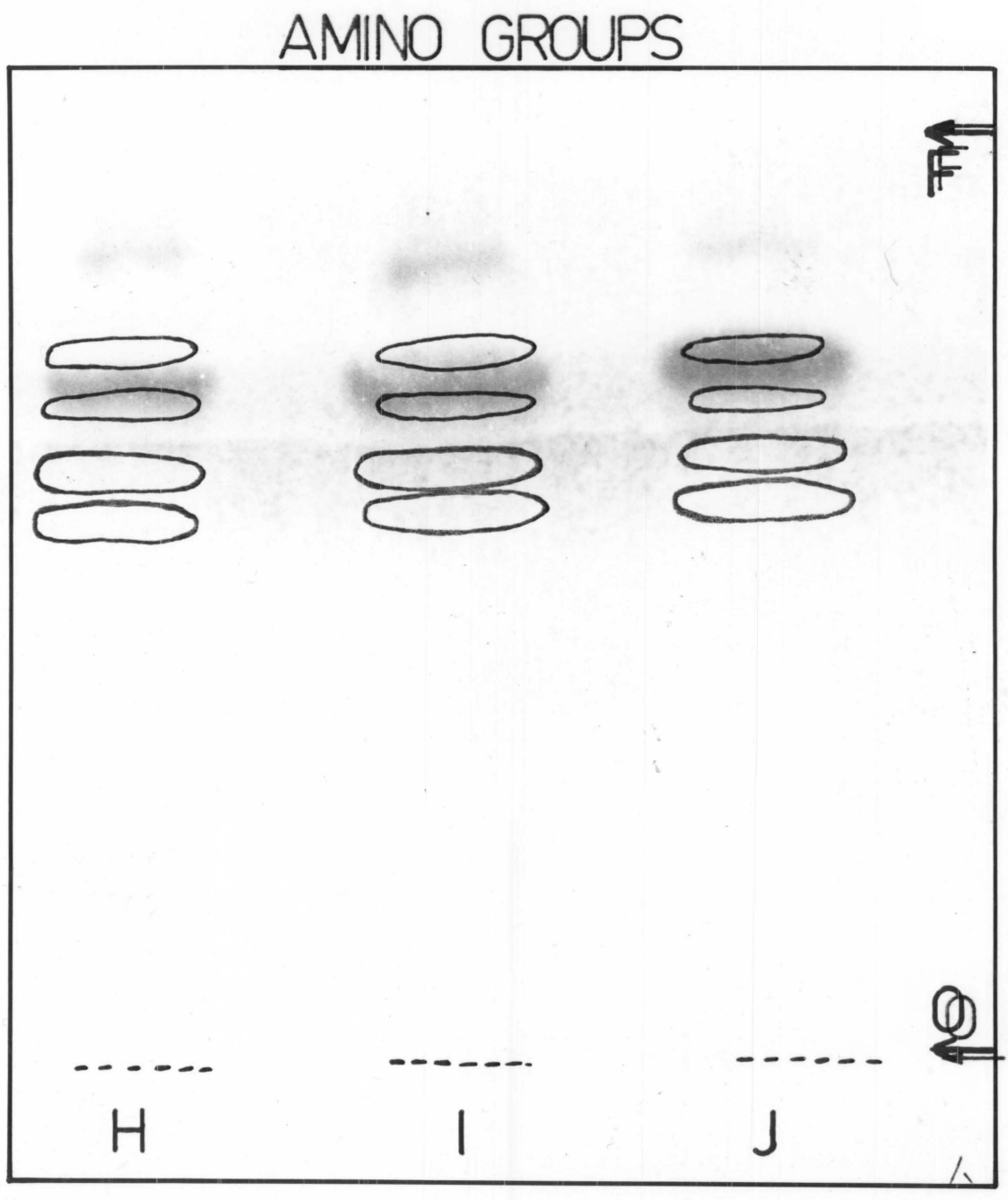
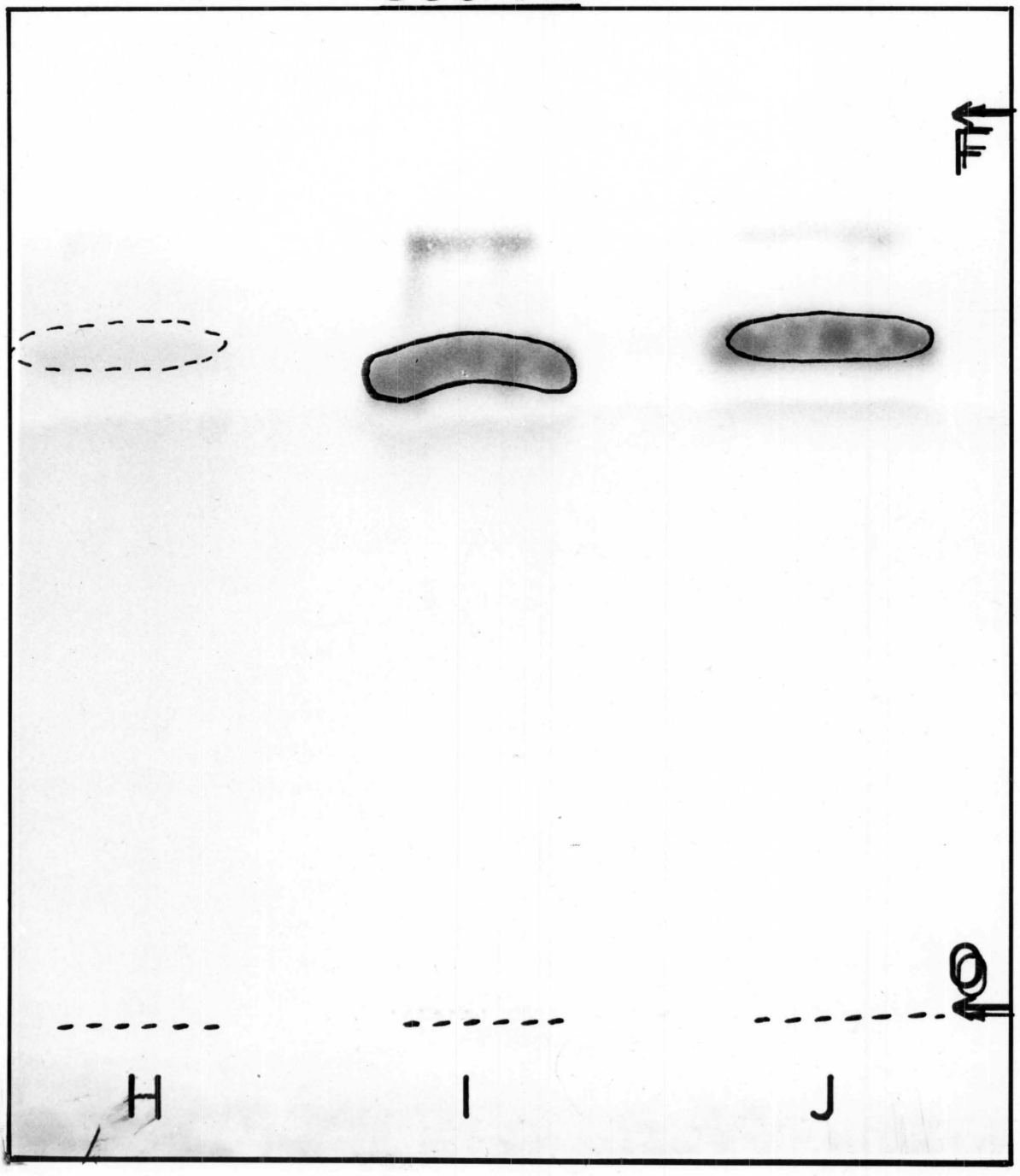

AMINO GROUPS
H
I
J

Figure 3.28: Legend as in Fig. 3.27 except analysed for radioactivity and sugars

(NB Sample H very faint because extract exhausted in the course of application to the TLC plate).

SUGARS



right across the autoradiograph and was not just confined to the regions containing the samples, it was considered an artefact, possibly due to reaction between the X-ray film and residual formic acid in the developing solvent.

The radioactivity in the main spot co-chromatographed with material that gave a positive sugar reaction (Fig 3.28). This material also fluoresced blue-white under UV and charred when sprayed with conc H_2SO_4 and heated to 100°C. Part of the radioactivity in the main spot was also associated with substances that gave an orange colour with ninhydrin (Fig. 3.27). Three other ninhydrin-positive spots (1 orange, 2 purple) were present but did not coincide with the radioactive or carbohydrate-containing areas on the TLC plate.

In both solvent systems, the radioactive/carbohydrate spot had a very faint yellow colour, which turned dark brown after the plates were stored in darkness for a number of days.

3.3.5.5 TLC of 'crude ester' fraction from untreated grains

TLC was also performed on acetone-precipitated material, obtained following extraction with 50% acetone from macerated grains which had not been subjected to injection. Aliquots of the radioactive aqueous extract from intact grains were run on each TLC plate for comparative purposes. The chemical and chromatographic properties of the untreated sample were identical to those of the labelled bound complex.

3.3.5.6 TLC of radioactive aqueous extract following hydrolysis

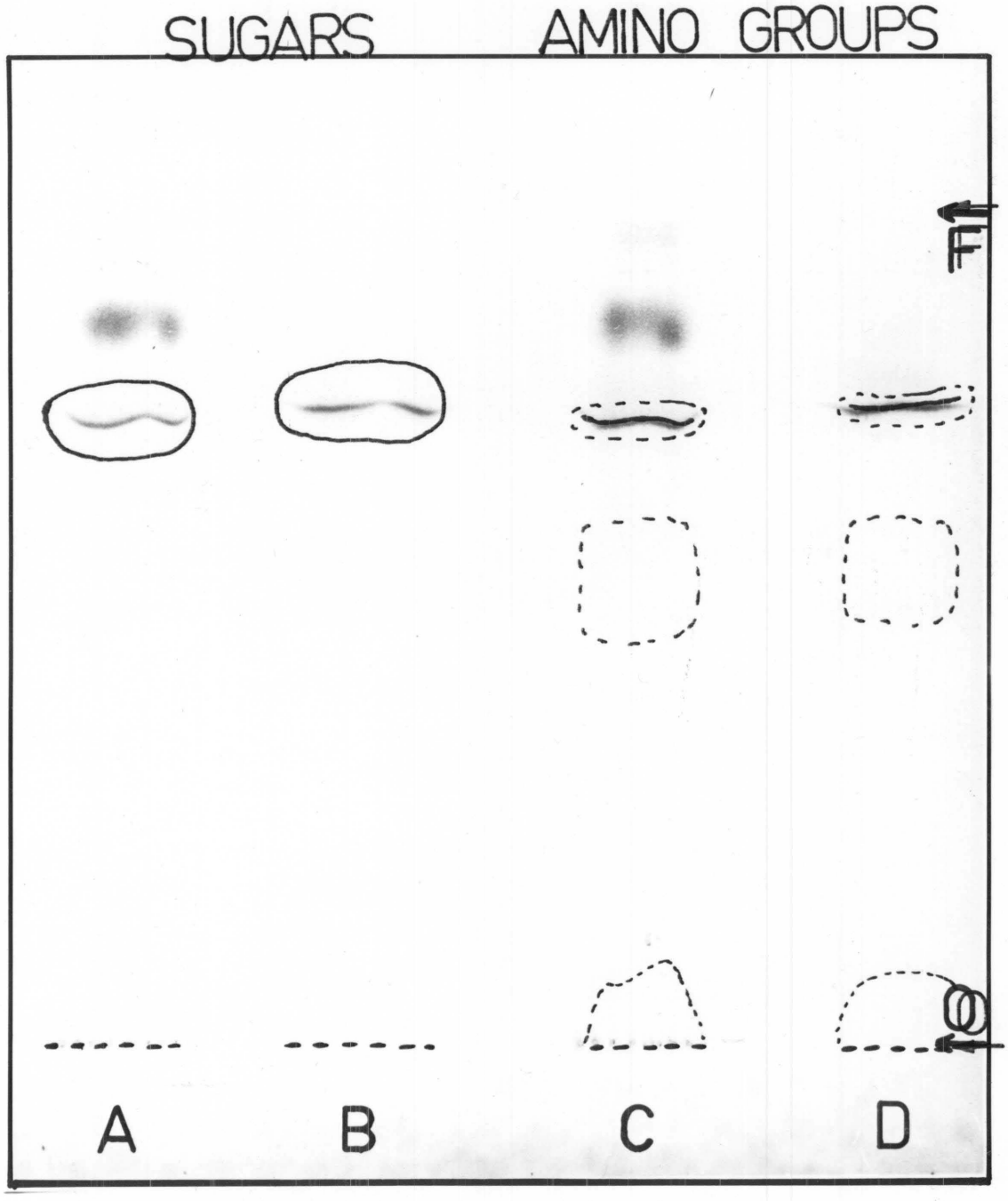
Aliquots of the labelled aqueous extract were hydrolysed in 1N NaOH for 1 h at 24°C, neutralised with H_2SO_4 and chromatographed in $CHCl_3/MeOH/H_2O$, together with unhydrolysed samples for comparative purposes. Following autoradiography, the radioactivity in the main zones was determined by LSA. Radioactivity was released from the bound complex by hydrolysis, mostly as a single spot, Rf 0.84 - 0.90 (Fig. 3.29). Table 3.6 shows that in untreated and hydrolysed extracts, the bound complex contained 73.2% and 27.6% of the radioactivity

Figure 3.29: TLC of alkali-hydrolysed aqueous extract in $CHCl_3/MeOH/H_2O$

One half of the plate (samples A and B) was analysed for sugars; the other for amino groups (Samples C and D). For details see text.

A and C. Hydrolysed

B and D. Control (unhydrolysed).


SUGARS
AMINO GROUPS
F
O
A
B
C
D

respectively, indicating that hydrolysis released 62.3% of the bound radioactivity. Eighty-two percent of the radioactivity liberated was located at Rf 0.84 - 0.90. The mobility of this spot, relative to the bound complex (R_{BOUND}), was 1.17, which was identical to the R_{BOUND} value observed for the lower faint band of radioactivity in Figs. 3.23 and 3.24. The remaining 18% of the liberated activity was situated near the solvent front. No shift in the position of the

Table 3.6: Silica gel scrapings of chromatogram as in Fig. 3.29 - % distribution of radioactivity

Mean of two samples

Spot	Untreated (%)	Hydrolysed (%)
bound complex (Rf 0.70 - 0.79)	73.2 ± 2.1	27.6 ± 0.1
Rf 0.84 - 0.90	10.4 ± 2.1	48.4 ± 0.9
Rf 0.95 - 1.0	-	8.3 ± 0.6
Remainder of plate	16.4 ± 1.0	15.7 ± 0.3
	100	100

diphenylamine/aniline- and ninhydrin-positive spots was apparent after hydrolysis. These observations were confirmed by TLC in the alternative solvent system (Fig. 3.30).

On the basis of TLC mobility and co-chromatography with authentic IAA-^{14}C (Fig. 3.31), the main labelled compound released was identified as IAA.

3.3.5.7 Visualisation of indoles on TLC

Renewed efforts were made to establish the presence of IAA in the endogenous bound complex. Sephadex-purified aqueous extracts from injected and untreated grains, were chromatographed and indoles visualised with the Salkowski reagent. A pink spot was revealed at Rf 0.25 - 0.33 in the $CHCl_3$/MeOH/H_2O solvent system. This was found to be the Ehmann-positive compound(s) which partially co-chromatographed with the bound complex during Sephadex G-25 chromatography.

Figure 3.30: TLC of hydrolysed aqueous extract in EtOAc/EtOH/HCOOH/H_2O.

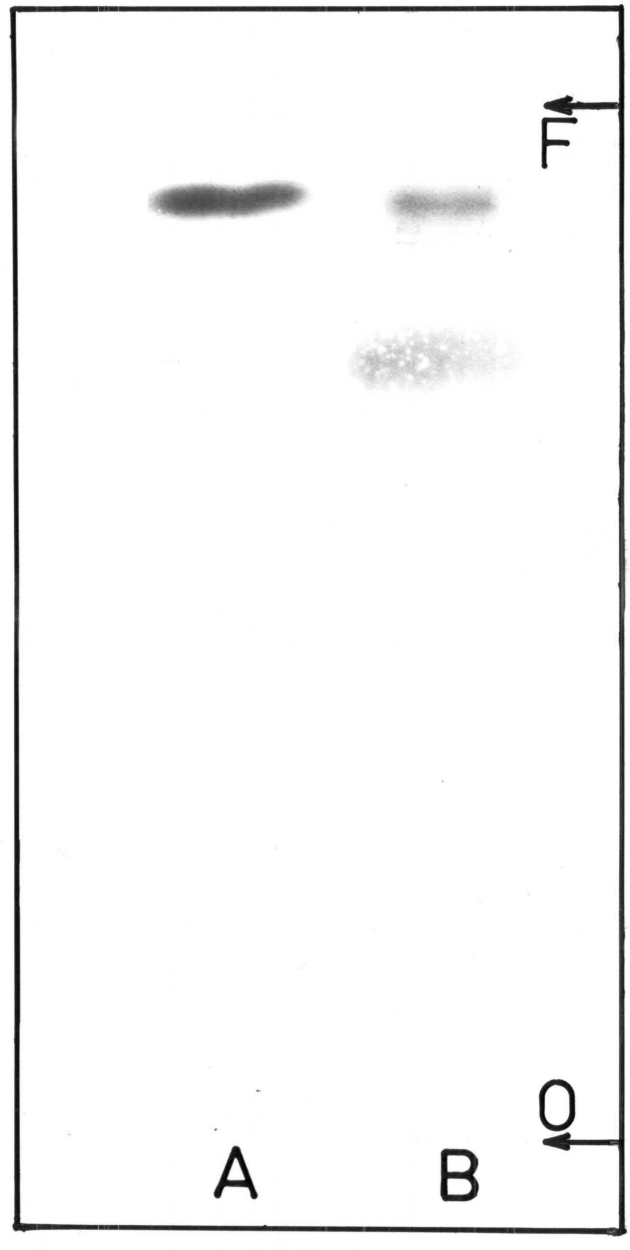
A. Hydrolysed, sugar detection
B. Hydrolysed, amino group detection
C. Control (not hydrolysed)


SUGARS
AMINOS
F
O
A
B
C

Figure 3.31: TLC of authentic IAA-^{14}C and hydrolysed aqueous extract in EtOAc/EtOH/HCOOH/H_2O

A. IAA-^{14}C

B. Hydrolysed extract


F
O
A
B

Detection of indole substances within the bound complex itself was complicated by the presence of the carbohydrate. This charred when the plates were heated after spraying with Salkowski reagent, due to the conc H_2SO_4 in the reagent mixture, thus masking any possible indole colour development in the spot.

3.3.5.8 Ammonolysis of the bound complex

This technique has the advantage that IAA is released from the bound complex, thus enabling detection using TLC spray reagents. In addition, if the IAA is bound by an ester linkage, indole-3-acetamide is characteristically produced (Percival and Bandurski, 1976).

(i) *Untreated grains.* Sephadex-purified water extracts from 30 non-injected grains, taken from 63 h old seedlings, were chromatographed on silica gel TLC plates developed with $CHCl_3$/MeOH/H_2O. The UV absorbing zones (Rf 0.60 - 0.75) were scraped from the plate, eluted with water, dried and finally hydrolysed in 2 ml of 28% NH_4OH (v/v) at 100°C for 1 h. Prior to heating, the samples were flushed with, and sealed under N_2. Following ammonolysis, the extracts were acidified to pH 2.5 and partitioned three times against ether. The combined ether extracts were dried, taken up in a small amount of ethanol and applied in bands to TLC plates. Authentic IAA was run in parallel to the samples. As a further standard, IAA was spotted on top of half of one band of ammonolysed sample. Duplicate chromatograms were prepared and developed with either iPA or BAW, and tested for indoles.

Two Salkowski-positive spots were apparent in the ammonolysed samples (Table 3.7). Authentic IAA run in parallel, did not correspond with either of these spots. However, IAA spotted on top of the sample partially co-chromatographed with the lower spot in iPA, and the more mobile spot in BAW (Table 3.7).

To provide a more accurate internal standard, ether extracts of the ammonolysed sample were spiked with a small quantity of IAA-^{14}C, thoroughly mixed and chromatographed on silica gel plates with iPA. After visualisation of the

Table 3.7: Mobility on TLC of ether-extractable compounds, following ammonolysis of the aqueous fraction from grains which had not been injected

Spot	Solvent	
	iPA	BAW
Salkowski-positive spots	Rf 0.32 - 0.35 & 0.62 - 0.68	Rf 0.61 - 0.66 & 0.68 - 0.74
Authentic IAA run in parallel	Rf 0.37 - 0.42	Rf 0.72 - 0.78
Authentic IAA spotted on top of ammonolysed sample	Rf 0.34 - 0.38	Rf 0.70 - 0.76

indoles with Salkowski reagent (Fig. 3.32), the silica gel in the region of the putative IAA (i.e. lower) spot, was carefully scraped from the plate and the radioactivity determined by LSA (Table 3.8).

Table 3.8: Radioactivity in silica gel bands, following TLC of aqueous fraction extracted into ether and spiked with IAA-^{14}C

See Fig. 3.32 for position of bands.

Region of plate	Designation	Radioactivity (Bq)	% of total radioactivity
putative IAA spot	A	331.1	79.2
4 mm zone below putative IAA spot	B	17.6	4.2
4 mm zone above putative IAA spot	C	37.8	9.0
4 mm zone above C	D	6.3	1.5
rest of plate		25.5	6.1
		418.3	

The bulk of the IAA-^{14}C (79.2%) was recoverable from the lower Salkowski-positive spot and 92.4% recovery was obtained when 4 mm zones either side of the spot were counted. The remaining radioactivity located elsewhere on the plate, represented the typical level of background activity. Thus, the lower Salkowski-positive spot was identified as IAA.

The other Salkowski-positive spot was tentatively identified as indole-3-acetamide, on the basis of Rf and R_{IAA} values. (Zenk, 1961; Stahl, 1969; Mousdale and Ward, 1981)

(ii) *Injected grains.* A quantity of radioactive bound sample, equivalent to the extract of 3 grains, was ammonolysed, extracted with ether and chromatographed as described above. Most of the radioactivity released from the bound complex was in the form of IAA-^{14}C (Fig 3.33). Only 4.8% was released as indole-3-acetamide, indicating that only a small amount of

Figure 3.32: Position of silica gel bands in Table 3.8

TLC in iPA of 'cold' grain extract spiked with IAA-^{14}C following ammonolysis, and visualised with Salkowski reagent.

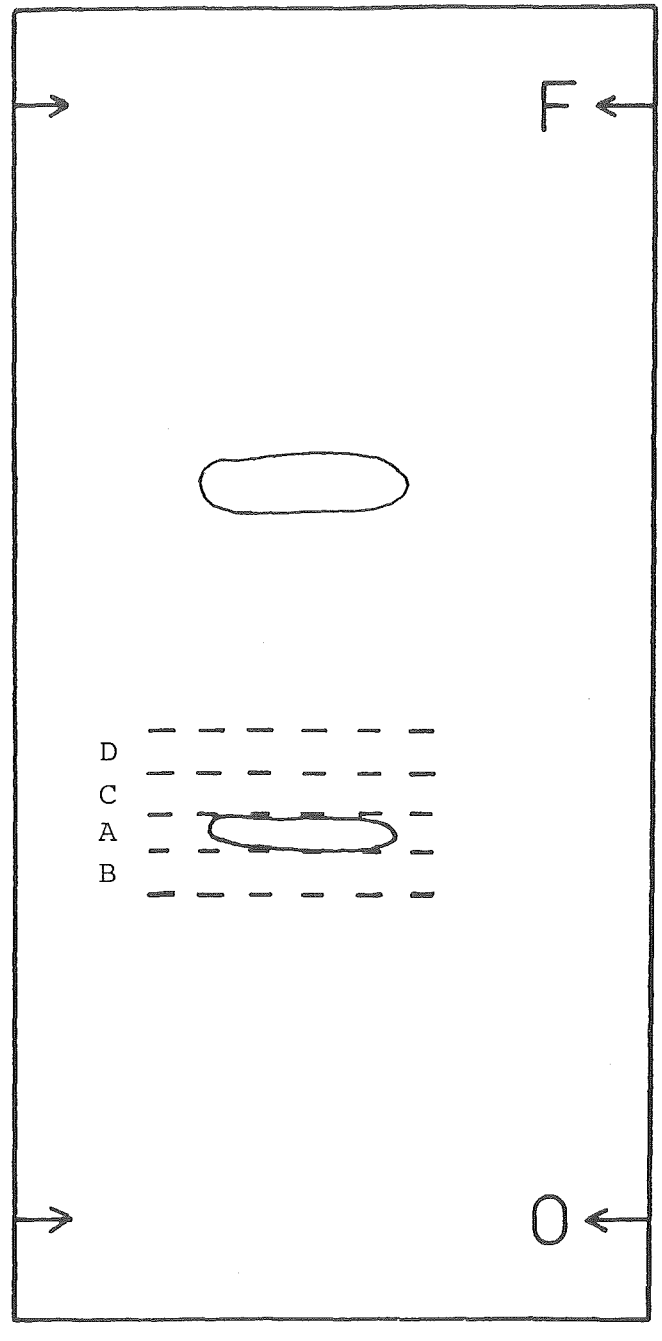
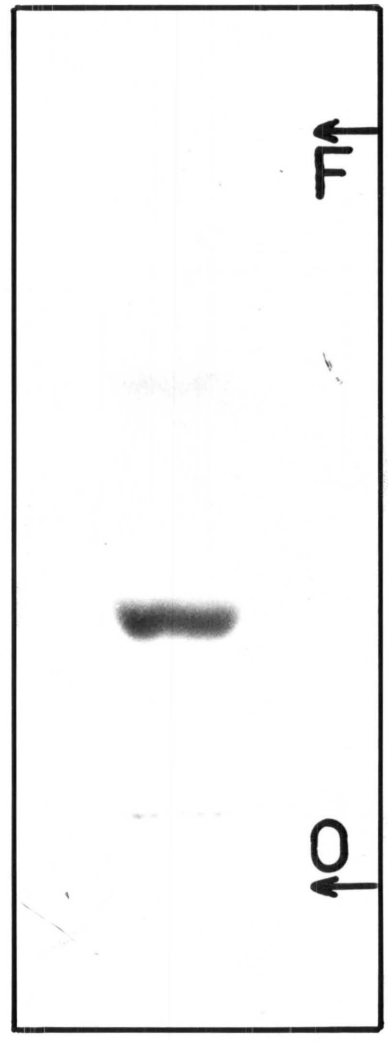

F
D
C
A
B
0

Figure 3.33: TLC in iPA of radioactive aqueous extract following ammonolysis and extraction into ether

Detection by autoradiography.


F
O

the radioactivity was bound via an ester linkage. The band of radioactivity at Rf 0.1 was not identified.

3.3.6 Comparison of ethanol- and acetone-based extraction methods

It was shown earlier that the 'bound' complex could be obtained from grains either by water extraction, or by precipitation, following 50% acetone extraction. Further experiments were carried out to determine the distribution of label between the various extractants (i.e. organic, water, NaOH and Soluene), depending on which organic solvent (i.e. 95% ethanol or 50% acetone) was used initially.

3.3.6.1 Extraction of whole grains

Fifty grains of *Avena* seedlings were injected with 2 kBq IAA-2-^{14}C. After 6 h incubation, the grains were extracted in either 95% ethanol or 50% acetone (5 grains per vial, 5 vials per solvent)(sections 2.5.1.3 and 2.5.4.1), followed by 2 d in distilled water, 40 h in 1N NaOH and finally overnight in Soluene. Aliquots of each fraction were assayed by LSA. Table 3.9 shows that 50% acetone was a more effective initial solvent and extracted 76.4% of the radioactivity from the grains, compared to 45.6% for 95% ethanol. Thus, much of the 95% ethanol-insoluble but water-soluble radioactivity, and part of the NaOH-soluble activity, was soluble in 50% acetone.

An aliquot of the 50% acetone extract was subjected to acetone precipitation. Table 3.10 shows that 21% of the activity was precipitable by high acetone concentrations. It has been shown that this material consisted of the 'bound' complex. Sephadex and TLC analysis of the water-soluble, but 50% acetone-insoluble fraction showed that 'bound' material was also present in this fraction - indicating that 50% acetone was only partially successful in extracting the 'bound' radioactivity from whole grains. According to Bandurski (*pers. comm.*), radioactivity diffusing into the body of the endosperm may become 'difficultly extractable" and thus be misidentified as 'bound'.

Table 3.9: Influence of initial solvent-type on distribution of radioactivity between fractions extracted from whole grains

For other details see text.

Initial solvent		Soluble in initial solvent	Water soluble	NaOH soluble	Residue (Soluene)	Total
95% Ethanol	Bq	2838 ± 312	2149 ± 206	992 ± 154	249 ± 19	6228 ± 691
	%	45.6	34.5	15.9	4.0	
50% Acetone	Bq	4501 ± 453	575 ± 111	740 ± 106	79 ± 16	5895 ± 686
	%	76.4	9.8	12.5	1.3	

Table 3.10: Distribution of radioactivity following acetone precipitation of 50% acetone-extract of intact grains

fraction	radioactivity (Bq)	% of total activity
precipitate	497	21.0
supernatant	1870	79.0
Total	2367	

3.3.6.2 Extraction of macerated grains

Further experiments were carried out in which the grains were disintegrated at termination of the experiments to aid extraction. This 'flour' was extracted initially with either 95% ethanol or 50% acetone (three 12 hourly changes), followed by extraction in water, NaOH and Soluene (section 2.5.4.2), and counted as before. The results are shown in Table 3.11 (data from Table 3.9, for whole grains, are shown for comparison). Macerating the grains prior to extraction altered the distribution of radioactivity between the four solvents in each extraction schedule. Thus, much more of the labelled material was extracted by the initial organic solvent. In the case of the 50% acetone-initiated system, over 93% of the total radioactivity in the macerated grains was extractable by 50% acetane, and only minimal amounts remained for extraction in water, NaOH and Soluene. By comparison, only 76% of the radioactivity in whole grains was extracted by 50% acetone.

Similarly, 95% ethanol extracted 73% of the activity from macerated grains, compared to 46% from whole grains. Twenty percent was extracted with water and only 5.4% with NaOH. Thus, 'bound' material, normally extracted in the water and NaOH fractions, was extracted by the initial organic solvent, when grains were macerated prior to extraction.

Table 3.11: Extraction of radioactivity from macerated grains

Details in text (3 replicate vials used). The percentage distribution of radioactivity for whole grains is shown for comparison.

Initial Solvent			Soluble in initial solvent	Water soluble	NaOH soluble	Residue (Soluene)	Total
95% Ethanol	macerated	Bq	3802 ± 190	1040 ± 165	282 ± 54	70 ± 6	5194 ± 415
		%	73.2	20.0	5.4	1.3	
	whole	%	45.6	34.5	15.9	4.0	
50% Acetone	macerated	Bq	4721 ± 407	132 ± 29	148 ± 14	54 ± 7	5055 ± 457
		%	93.4	2.6	2.9	1.1	
	whole	%	76.4	9.8	12.5	1.3	

(i) *'Free' and hydrolysable radioactivity determinations.* Aliquots of the 95% ethanol and 50% acetone solutions were taken to determine the amounts of 'free' and hydrolysable radioactivity in the extracts.

For 'free' radioactivity determinations, 2 ml samples were dried, taken up in 1.5 ml water, acidified to pH 2.5 and extracted 3 times with 1 ml ether. To determine 'bound' radioactivity, similar samples were dried, hydrolysed in either 1.5 ml 1N NaOH for 1 h at 24°C or 1 ml 7N NaOH for 3 h at 100°C, and then acidified and extracted with ether (as above), giving 'free' plus 'hydrolysable' radioactivity. Duplicate samples were run for each treatment.

The radioactivity in the aqueous phase was assayed directly by LSA, and 1 ml aliquots were taken for the ether determinations. The results, after correction for the volume sampled, are shown in Table 3.12. The remainder of the ether solutions were analysed by TLC.

In the 95% ethanol and 50% acetone extracts, an average of 36.6% and 27.2% of the radioactivity, respectively, was acid-ether soluble. This radioactivity, most of which co-chromatographed with IAA in both iPA (Fig. 3.34) and MEK/hexane (Fig. 3.35), amounted to 26.8% (36.6 x 73.2%; Table 3.11) of the total radioactivity extracted from the macerated grains by the 95% ethanol-based extraction system, and 25.4% of the total in the acetone system.

Mild alkaline hydrolysis of the 95% ethanol extract liberated a small (3.7%), but significant amount (Student's t test, $P < 0.05$) of additional ether soluble radioactivity, also in the form of IAA (Fig. 3.36b). In the 50% acetone extract mild hydrolysis released IAA-^{14}C amounting to 27.9% of the radioactivity in the extract, or 26.1% of the total activity in the grain (Table 3.12 and Fig. 3.36a).

Hydrolysis with strong base at elevated temperatures resulted in the appearance of additional labelled compounds in the ether phase (Fig. 3.37). However, since this treatment resulted in substantial losses of radioactivity, it was impossible to tell whether these compounds were liberated from the 'bound complex' by hydrolysis or were breakdown products of IAA-^{14}C.

Table 3.12: Estimation of 'free' and hydrolysable radioactivity

See text for details.

Extract	Treatment		Radioactivity (Bq)			% in ether	Average (%)
			Aqueous	Ether	Total		
95% ethanol	not hydrolysed	i	248.9	138.8	386.7	35.9	36.6
		ii	251.3	148.8	400.1	37.2	
	hydrolysed, 1N NaOH	i	230.7	159.7	390.4	40.9	40.3
		ii	229.7	150.6	380.3	39.6	
	hydrolysed, 7N NaOH	i	160.9	139.1	300.0	46.4	40.0
		ii	206.6	104.7	311.3	33.6	
50% acetone	not hydrolysed	i	338.4	131.0	469.4	27.9	27.2
		ii	338.9	120.9	459.8	26.3	
	hydrolysed, 1N NaOH	i	201.3	239.2	440.5	54.3	55.1
		ii	200.3	253.0	453.3	55.8	
	hydrolysed, 7N NaOH	i	162.5	191.6	354.1	54.1	53.5
		ii	183.3	205.8	389.1	52.9	

Figure 3.34. TLC of 'free' radioactivity in iPA

Detection was by autoradiography. For other details see text.

A. Authentic IAA-^{14}C
B. 50% acetone extract
C. 95% ethanol extract

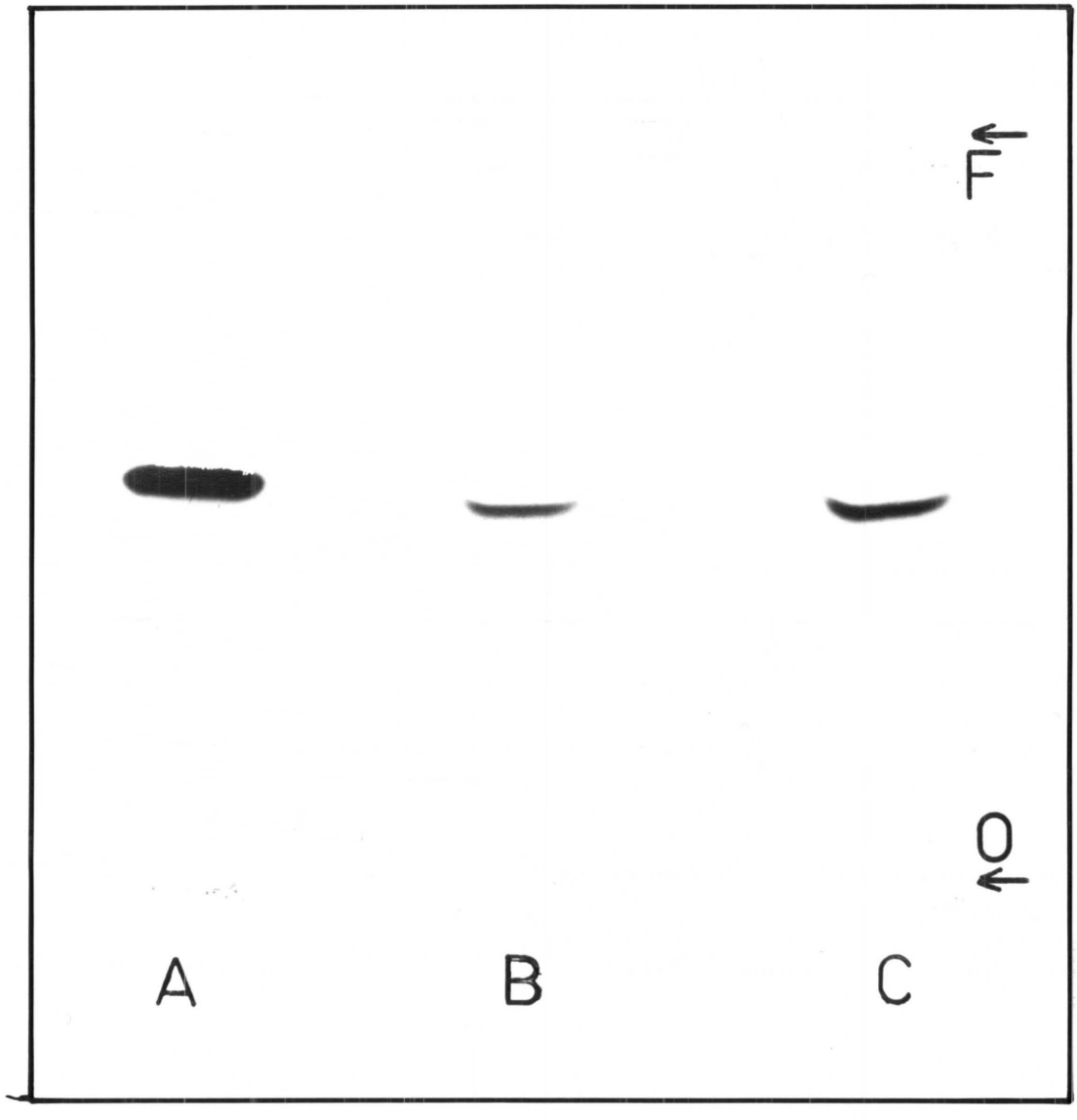

F
O
A
B
C

Figure 3.35: TLC of 'free' radioactivity in MEK/hexane

Detection was by autoradiography. For other details see text.

A. Authentic IAA-^{14}C
B. 50% acetone extract
C. 95% ethanol extract

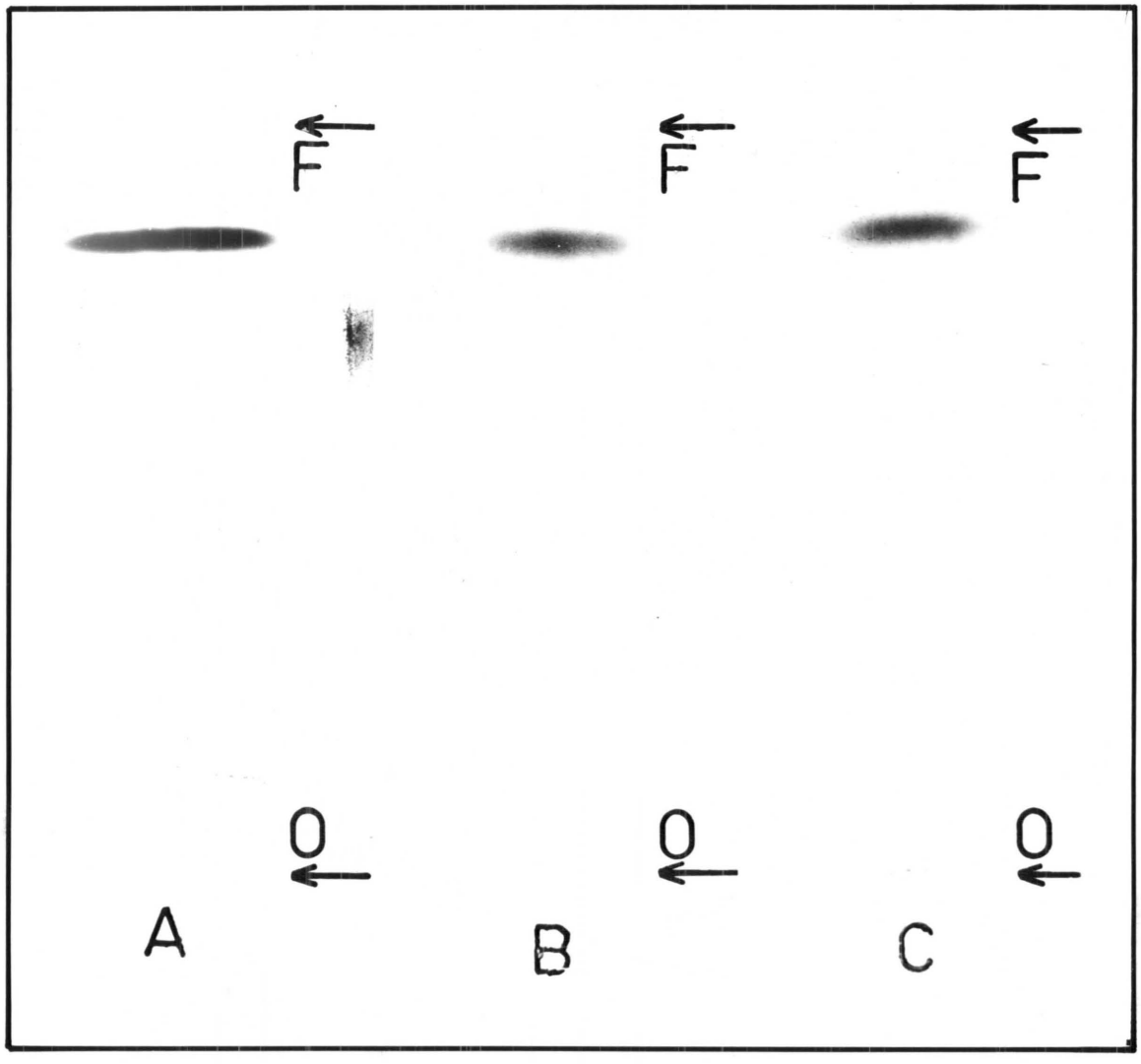

F
F
F
O
O
O
A
B
C

Figure 3.36: TLC of 'free' plus 1N NaOH-hydrolysable radioactivity in iPA

Dectection was by autoradiography. For other details see text.

A. 50% acetone extract
B. 95% ethanol extract

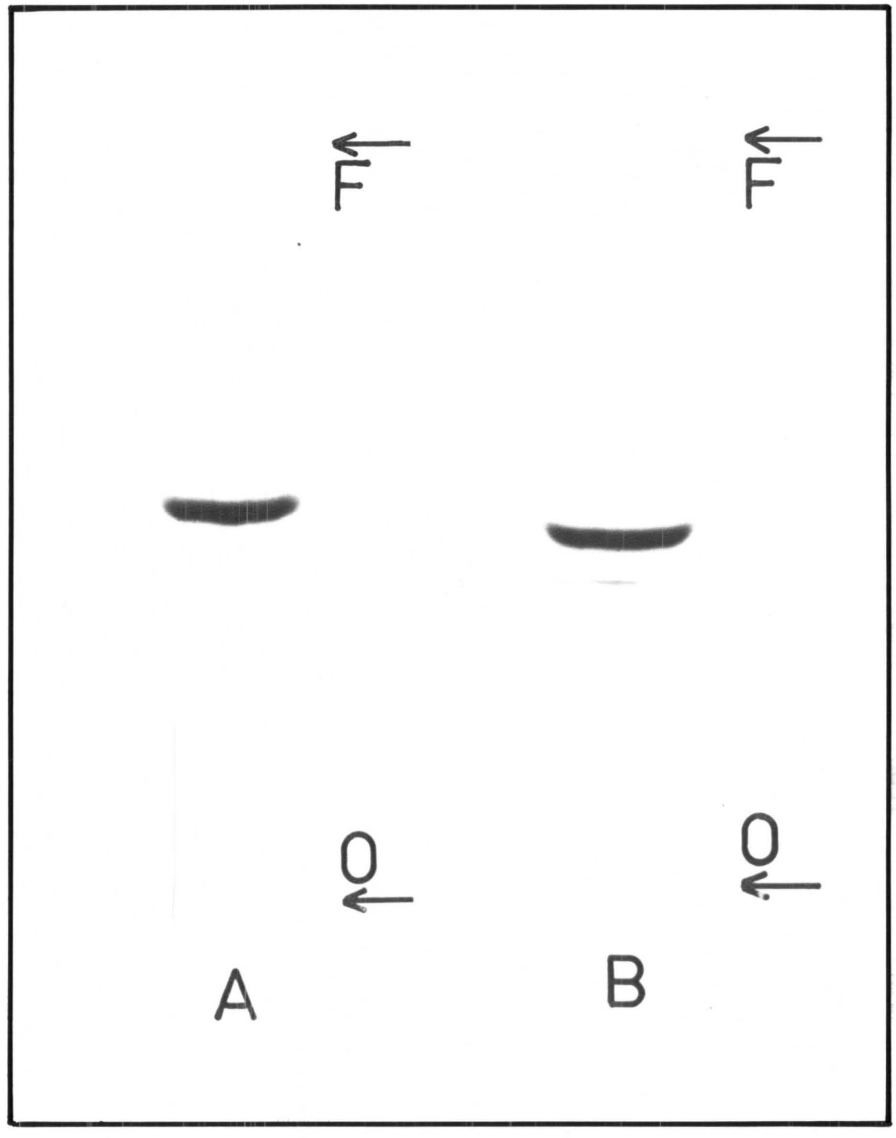
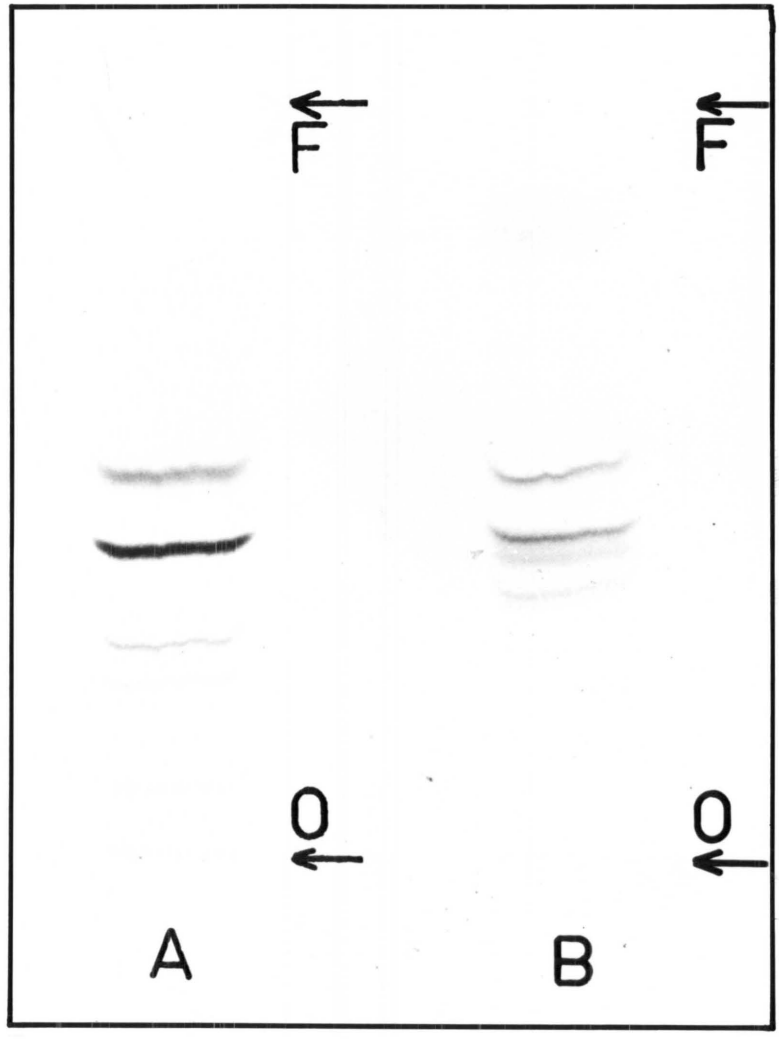

F
F
O
O
A
B

Figure 3.37: TLC of 'free' plus 7N NaOH-hydrolysable radioactivity in iPA

Detection was by autoradiography. For other details see text.

A. 50% acetone extract
B. 95% ethanol extract


F
F
O
O
A
B

(ii) *Acetone precipitation.* Acetone precipitation of 10 ml aliquots of the 95% ethanol and 50% acetone extracts resulted in 6.9% and 33% respectively, of the radioactivity being precipitated. This represented 5.1% of the total radioactivity extracted by the ethanol-initiated extraction schedule and 30.1% of that extracted by the acetone system.

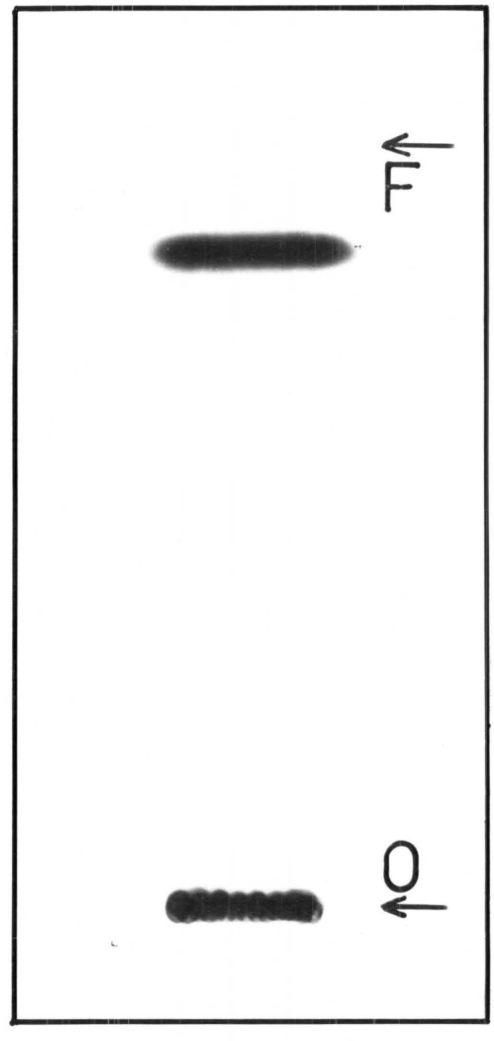
(iii) *Nature of labelled compounds extracted following maceration.* With respect to the ethanol-based extraction method, the above results together indicated that only a small part of the 'water-soluble' material that became extractable with 95% ethanol as a result of maceration, was reversibly-bound IAA or material that was precipitable by acetone. However, it was established earlier that almost all of the water-soluble, but ethanol-insoluble, material in intact grains was in the form of the 'bound complex' (e.g. section 3.3.5.4). Further experiments were therefore carried out to determine the nature of the labelled compounds, that became extractable with the organic solvents following maceration.

In addition,it was apparent that since the 'bound' radioactivity was soluble in 95% ethanol when grains were macerated, 95% ethanol may also extract this material from intact grains. Earlier experiments had established that 95% ethanol extracts of intact grains, harvested 6 h after injection, contained mainly IAA-^{14}C and one major metabolite (Fig. 3.38). IAA accounted for 62% of the radioactivity. The major metabolite, which gave a positive ninhydrin reaction, was immobile when chromatographed in both MEK/hexane or iPA, and contained 20% of the radioactivity. These properties suggested that the metabolite may be 'bound', and so the nature of this compound was also investigated further.

Following TLC in MEK/hexane, a band of silica gel from the origin was eluted with 50% acetone, dried and the radioactivity taken up in 50% aqueous ethanol (v/v). The purified metabolite was spotted onto a TLC plate, together with samples of the 95% ethanol extract supernatant and

Figure 3.38: TLC in MEK/hexane of 95% ethanol extract of intact grains, harvested 6 h after injection of 2 kBq IAA-^{14}C.

Detection by autoradiography.


F
O

precipitate, as well as 50% acetone supernatant, and for reference purposes an aliquot of known, water-soluble 'bound' radioactivity. After development in $CHCl_3/MeOH/H_2O$, the plate was sprayed with ninhydrin and then autoradiographed.

Fig. 3.39 shows that 95% ethanol extracted what appeared to be 'bound' material from intact grains. Further, 'bound' radioactivity remained in the supernatant of the 95% ethanol and 50% acetone extracts after acetone precipitation. These results suggested that the 'bound' labelled material comprised 2 distinct, although chromatographically identical, fractions - one that was soluble in 80-90% acetone, and one that was insoluble in 80-90% acetone and therefore precipitated.

The similar amounts of radioactivity that could be precipitated and that could be liberated by mild alkaline hydrolysis (Tables 3.10 and 3.12, and text, section 3.3.6.2) suggested these were common properties of one fraction - an acetone-insoluble, reversibly-bound IAA fraction. The other, 80-90% acetone-soluble, fraction was designated 'X' to differentiate it from the 'reversibly-bound IAA' fraction. 'X' chromatographed with ninhydrin - positive substances (Fig. 3.39).

From Fig. 3.39 it is apparent that the remainder of the radioactivity in the grain was concentrated in 2 ninhydrin-negative compounds. The most mobile spot co-chromatographed with authentic IAA. The other compound, designated 'Y', was present in the supernatant of both the 95% ethanol and 50% acetone extracts, and formed the bulk of the 'immobile metabolite' fraction (i.e. the labelled material eluted from the origin following TLC with MEK/hexane). The remainder of this 'immobile' fraction included 'X' and a compound that ran near IAA, but which was not confirmed as IAA.

The radioactivity in the main spots of the chromatogram in Fig. 3.39 was assayed by LSA (Table 3.13), and these data, together with those from Tables (3.9 - 3.12), were used to estimate the proportions of radioactivity in each of the 4 main fractions i.e. IAA, bound IAA, and metabolites 'X' and

Figure 3.39: TLC in $CHCl_3/MeOH/H_2O$ of ^{14}C-labelled compounds extractable from *Avena* grains

Detection was by autoradiography and ninhydrin. For other details see text.

A. 95% ethanol extract supernatant
B. 95% ethanol extract precipitate
C. 'Immobile metabolite'
D. Aqueous extract
E. 50% acetone extract supernatant.

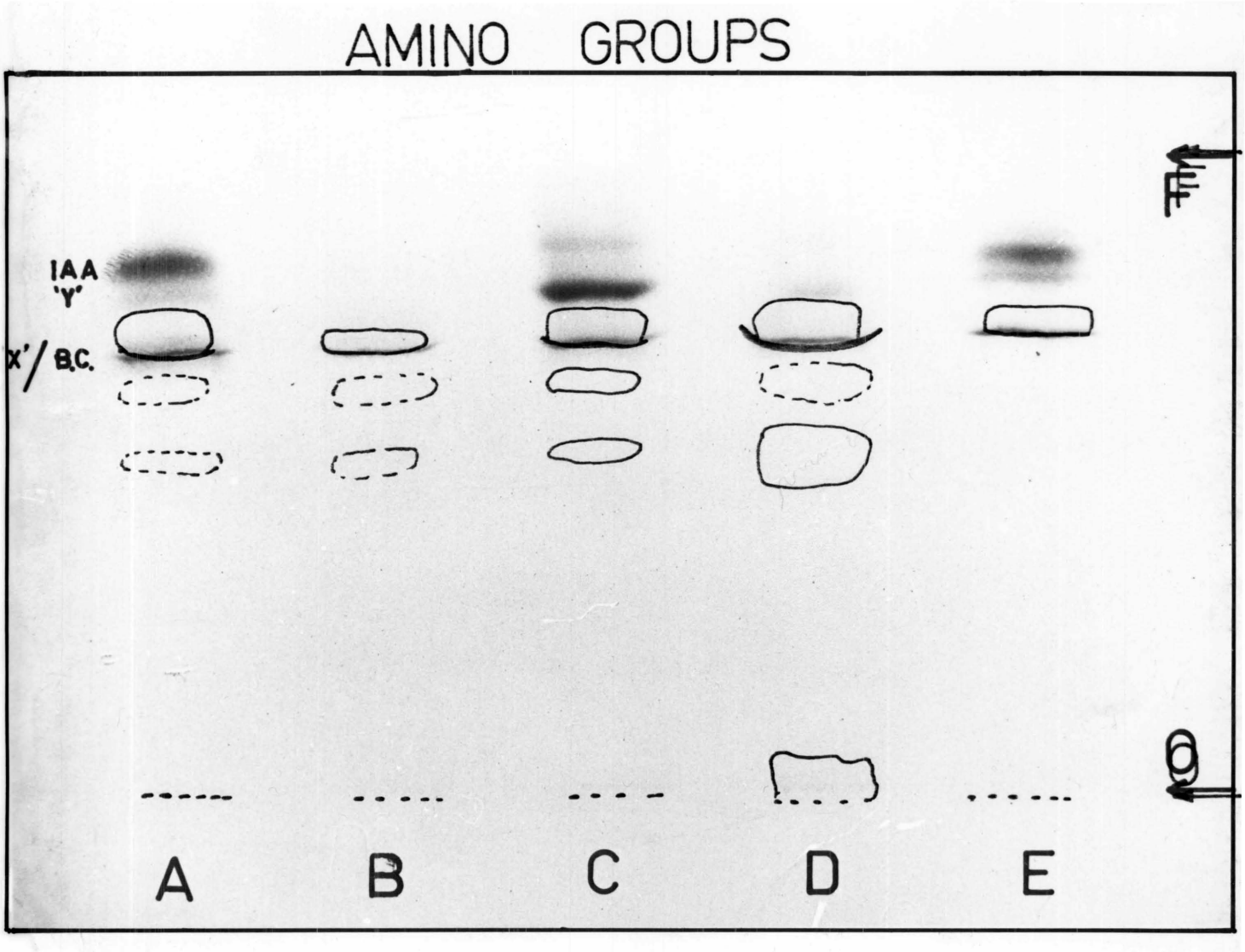

AMINO GROUPS
IAA
'Y'
X'/B.C.
A
B
C
D
E

Table 3.13: Radioactivity in silica gel scrapings from chromatogram shown in Fig. 3.39.

(The scrapings from sample B were accidentally spoiled).

Spot	Sample A Ethanol extract supernatant		Sample C Ethanol extract metabolite		Sample D Water-soluble bound extract		Sample E Acetone extract supernatant	
	Bq	%	Bq	%	Bq	%	Bq	%
IAA	13.4	41.2	8.6 IAA?	21.1	1.0	6.0	14.4	38.8
'Y'	4.2	12.8	21.5	53.0	2.6	15.6	6.4	17.2
'X'/ B.C. (Bound Complex)	11.2	34.5	8.6	21.1	11.3	68.1	12.6	34.1
Remainder of plate	3.7	11.5	2.0	4.8	1.7	10.3	3.7	9.9
Total	32.5		40.7		16.6		37.1	

'Y' (Table 3.14). The proposed interrelationship of the 4 fractions when the radioactivity in the grain was extracted using the different schedules is shown diagrammatically in Fig. 3.40.

The various estimations of the radioactivity in each fraction (Table 3.14) agree closely and indicate: that 6 h after injection of 2 kBq IAA-^{14}C, 24-28% of the radioactivity in the grain was as IAA, 26-30% as bound IAA, about 22% as 'X', and 10% as 'Y'.

Table 3.14: Estimates of radioactivity in IAA, bound IAA and metabolites 'X' and 'Y' fractions

Method of Estimation	%	Calculation
Free IAA		
(a) IAA spot from TLC of 95% ethanolic extracts, whole grains	28.3	IAA = 62% of ethanol extract 95% ethanol extract = 45.6% total radioactivity in grain (Table 3.9) 62 x 45.6% = 28.3%
(b) Ether extractable radio-activity in 95% ethanolic extracts, macerated grains	26.8	Ether extractable = 36.6% of 95% ethanol extract (Table 3.12) 95% ethanol extract = 73.2% of total radioactivity (Table 3.11) 36.6 x 73.2 = 26.8%
(c) TLC of supernatant, acetone precipitated 95% ethanol extract, macerated grains	28.1	IAA = 41.2% of supernatant (Table 3.13) supernatant = 93.1% of ethanol, extractable radio activity (section 3.3.6.2 ii) 95% ethanol extract = 73.2% of total activity (Table 3.11) 41.2 x 93.1 x 73.2 = 28.1%
(d) Ether extractable radio-activity in 50% acetone extracts, macerated grains	25.4	Ether extractable = 27.2% of 50% acetone extract (Table 3.12) 50% acetone extract = 93.4% of total activity (Table 3.11) 27.2 x 93.4 = 25.4%

Table 3.14 continued over page

Table 3.14 Continued

Method of Estimation	%	Calculation
(e) TLC of supernatant, acetone precipitated 50% acetone extract, macerated grains	24.3	IAA = 38.8% of supernatant (Table 3.13) Supernatant = 67% of acetone extract (section 3.3.6.2ii) 50% acetone extract = 93.4% of total radioactivity (Table 3.11) 38.8 x 67 x 93.4 = 24.3%
Bound IAA		
(a) Precipitated from 50% acetone extracts, macerated grains	30.1	precipitate = 33% of 50% acetone extract (section 3.3.6.2ii) 50% acetone extract = 93.4% of total activity (Table 3.11) 33 x 93.4 = 30.1%
(b) Released from 50% acetone extracts, (macerated grains) by 1N NaOH hydrolysis	26.1[1]	radioactivity liberated = 27.9% of 50% acetone extract (Table 3.12) 27.9 x 93.4 = 26.1%
(c) Precipitated from 95% ethanol extracts, macerated grains + water soluble and NaOH soluble radioactivity	30.6	precipitate = 6.9% of 95% ethanol extract 95% ethanol extract = 73.2% of total activity (Table 3.11) 6.9 x 73.2 = 5.1% Water + NaOH soluble activity = 25.4% (Table 3.11)

Table 3.14 continued over page

[1] The amount of 'bound IAA' estimated by acetone precipitation and by hydrolysis differed because not all of the radioactivity released by hydrolysis was ether soluble (cf Figs. 3.29 & 3.36).

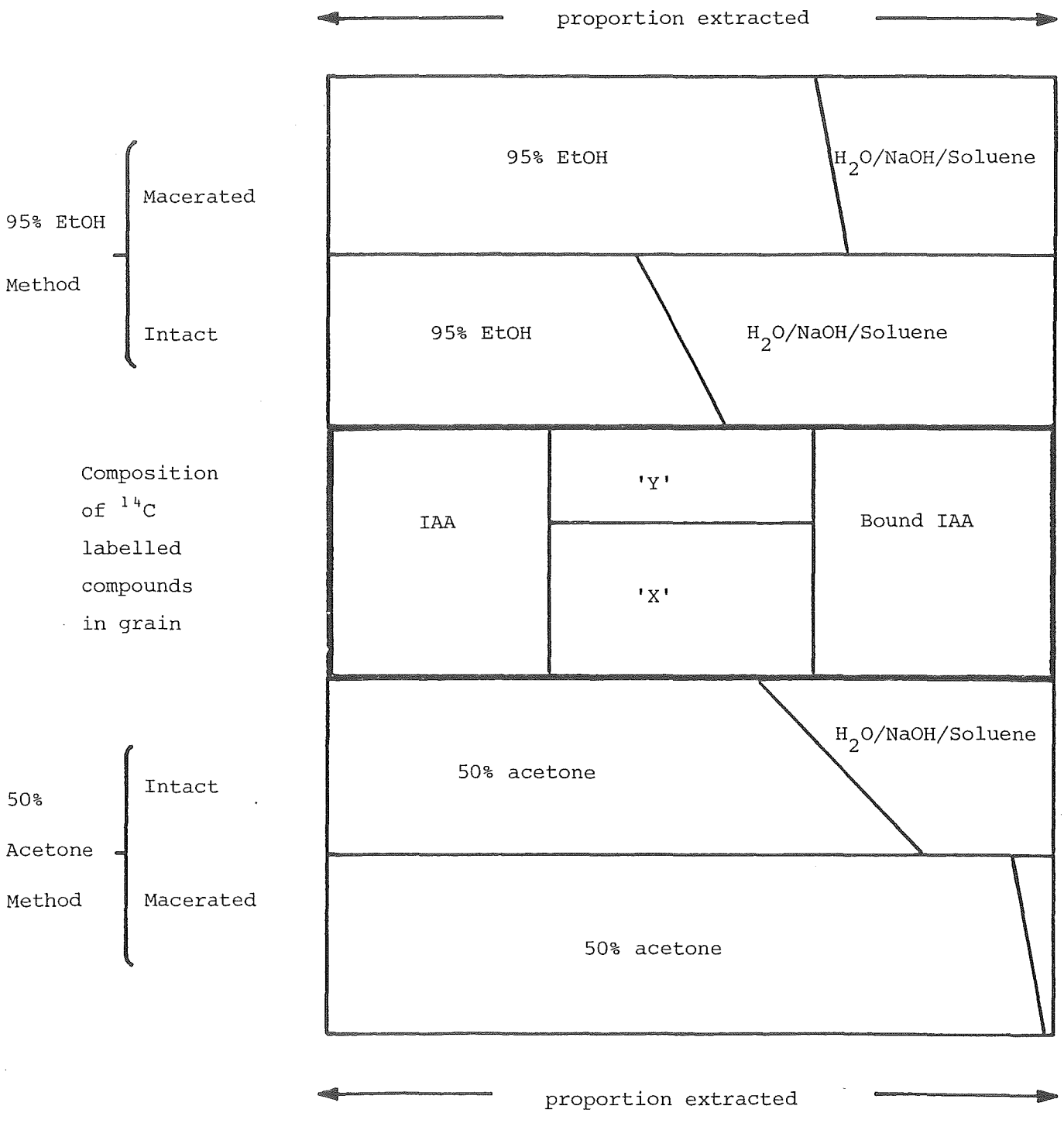
Table 3.14 Continued

Method of Estimation	%	Calculations
(d) Released from 95% ethanol extracts (macerated grains by 1N NaOH hydrolysis + water soluble & NaOH soluble radioactivity	28.1[1]	radioactivity liberated = 3.7% of 95% ethanol extract (Table 3.12) 3.7 x 73.2 = 2.7% + 25.4 (as above)
Metabolite 'X'		
(a) TLC of supernatant, acetone precipitated, 50% acetone extract, macerated grains	21.3%	'X' = 34.1% of supernatant (Table 3.13) Supernatant = 67% of 50% acetone extract 50% acetone extract = 93.4% of total activity 34.1 x 67 x 93.4 = 21.3%
(b) TLC of supernatant, acetone precipited, 95% ethanol extract, macerated grains	23.5	'X' = 34.5% of supernatant supernatant = 93.1% of 95% ethanol extract, 95% ethanol extract=73.2% of total activity 34.5 x 93.1 x 73.2 = 23.5%
Metabolite 'Y'		
(a) as for metabolite 'X' above (50% acetone)	10.8	'Y' = 17.2% of supernatant 17.2 x 67 x 93.4 = 10.8
(b) as for metabolite 'X' above (95% ethanol)	8.7	'Y' = 12.8% 12.8 x 93.1 x 73.2 = 8.7
(c) TLC of purified metabolite in Fig. 3.39 + radioactivity of some Rf in water soluble 'bound' extract from whole grains (also Fig. 3.39)	10.2	'Y' = 53% of 'purified metabolite' purified metabolite = 20% of 95% ethanol extract 95% ethanol extract = 45.6% of total activity 53 x 20 x 45 x 45.6 = 4.8% + 'Y'=15.4% of water soluble 'bound' (Table 3.13) water soluble bound = 34.5% of total activity 15.6 x 34.5 = 5.4

[1] 10-15% of the radioactivity in the grain remained unaccounted for by these estimates. Most of this comprised background radioactivity on TLC plates (e.g. Fig. 3.39).

Figure 3.40: Diagrammatic respresentation of proposed distribution of radioactivity between the four extractants when intact and macerated grains were extracted, using the 95% ethanol-based or 50% acetone-based extraction schedules.

Sloping lines indicate that the demarcation of extracted radioactivity between the solvents was not strict.


proportion extracted
95% EtOH
Macerated
95% EtOH
Method
Intact
95% EtOH
H_2O/NaOH/Soluene
95% EtOH
H_2O/NaOH/Soluene
Composition
of ^{14}C
labelled
compounds
in grain
IAA
'Y'
'X'
Bound IAA
50%
Acetone
Method
Intact
Macerated
50% acetone
H_2O/NaOH/Soluene
50% acetone
proportion extracted

3.4 RADIOTRACER STUDIES II

3.4.1 IAA-2-^{14}C injection experiments

The time-course experiments described in section 3.3.2 involved the extraction of whole grains by the ethanol extraction method. This method had been used successfully by a number of researchers to extract IAA-^{14}C and its derivatives from a variety of plant tissues (see section 4.3). Using extracts derived from intact Avena grains with this method, valid information on the nature of the ^{14}C-labelled metabolites formed in the grain was obtained in this investigation . However, the results detailed above indicated that partitioning by solvent extraction was not a suitable method for quantification of the various labelled fractions, since part of the 95% ethanol-soluble radioactivity was found to remain in the intact grain.

The time-course experiments were carried out prior to these observations, and it was impossible to differentiate retrospectively between the various IAA metabolites. However, in the 2 kBq injection experiments, aliquots of the 95% ethanol extracts of the grains, upper mesocotyl and coleoptile tip from all harvest times, were chromatographed, and the radioactivity in the major spots determined by LSA. Similar analysis was performed at the 6h harvest, in the 200 Bq and 20 Bq injection experiments. This allowed the amount of free IAA-^{14}C to be estimated. The total extractable radioactivity was therefore differentiated into free IAA and metabolised IAA (i.e. all the other fractions - bound IAA, 'X' 'Y' and the residue). Since the metabolism of IAA-^{14}C in the seedling was considered to be of primary importance and the nature of the metabolites of secondary importance, such a distinction was acceptable.

3.4.1.1 Distribution of radioactivity in the seedling

Figs. 3.41, 3.42 and 3.43 show the distribution of radioactivity on the various parts of the seedling, 1,3,6 and 24 h after injection into the endosperm of 2 kBq, 200 Bq and 20 Bq IAA-^{14}C. Although the amounts of IAA-^{14}C injected differed by one hundred-fold from one extreme to the other, a similar basic pattern of distribution of radioactivity was observed in each figure. This was characterised by a rate of transport from the grain into the shoot and roots, which

Figure 3.41: Time-course of distribution of radioactivity in seedlings following injection into the endosperm of 2 kBq IAA-^{14}C.

Values represent the mean ± standard error of 5 replicates each containing 5 items (where no standard error is shown the error term is less than or equal to the symbol used to designate the mean).

○ Grain
△ Shoot
□ Roots

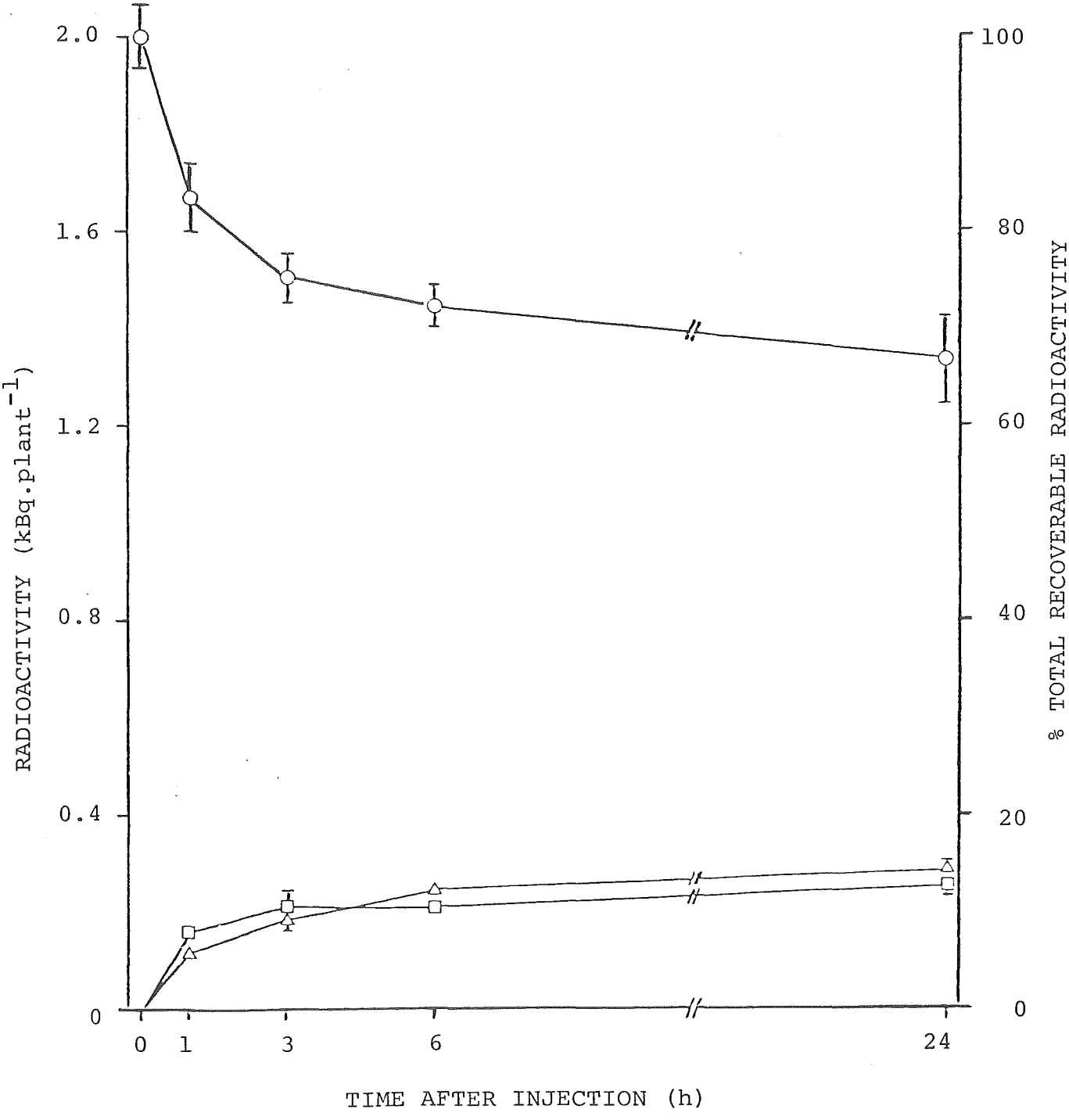
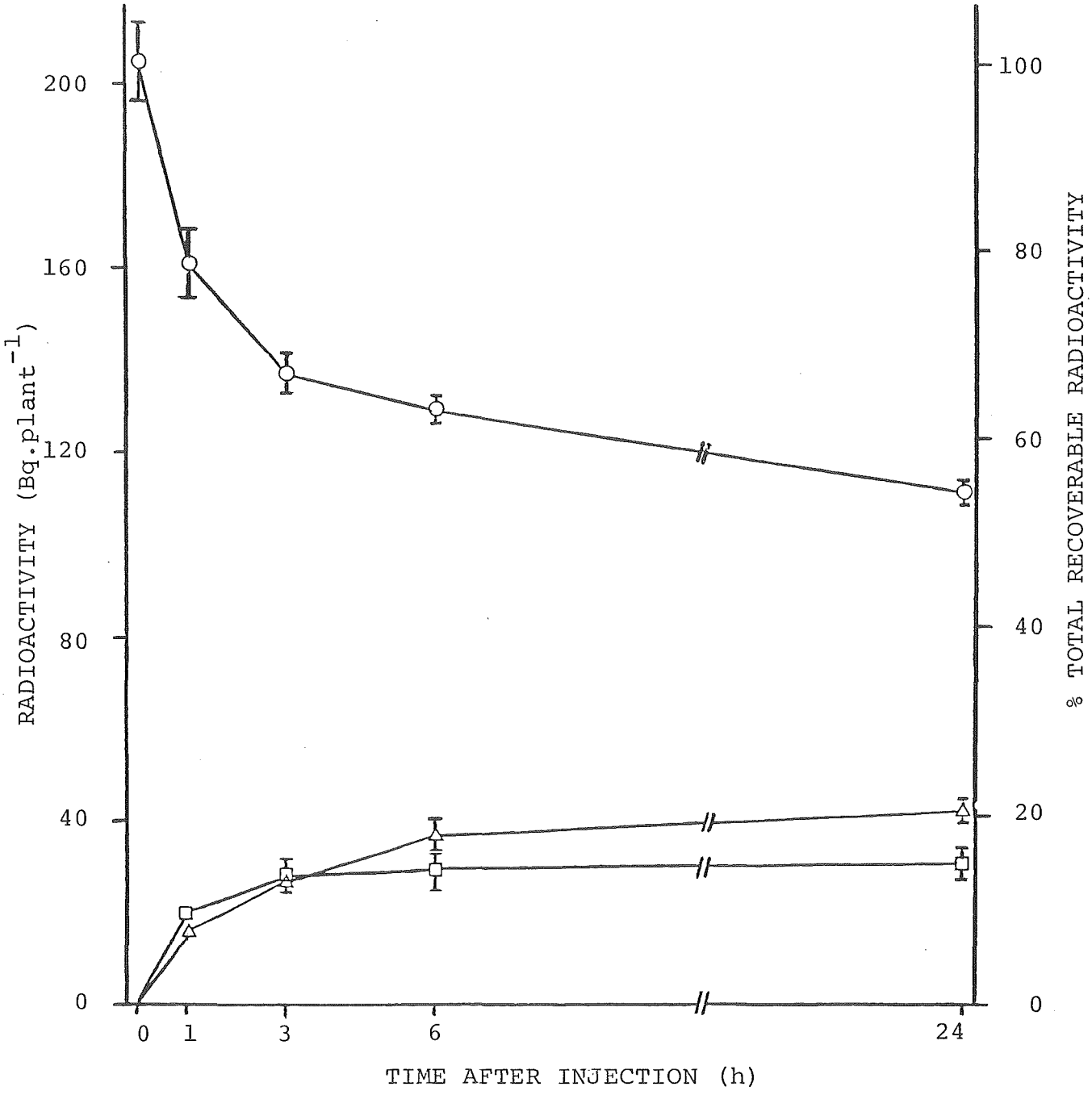

2.0
1.6
1.2
0.8
0.4
0
RADIOACTIVITY (kBq.plant^{-1})
100
80
60
40
20
0
% TOTAL RECOVERABLE RADIOACTIVITY
0
1
3
6
24
TIME AFTER INJECTION (h)

Figure 3.42: Time-course of distribution of radioactivity in seedlings following injection into the endosperm of 200 Bq IAA-^{14}C (n = 5)

○ Grain

△ Shoot

□ Roots


RADIOACTIVITY (Bq.plant^{-1})
% TOTAL RECOVERABLE RADIOACTIVITY
TIME AFTER INJECTION (h)
0
40
80
120
160
200
0
20
40
60
80
100
0
1
3
6
24

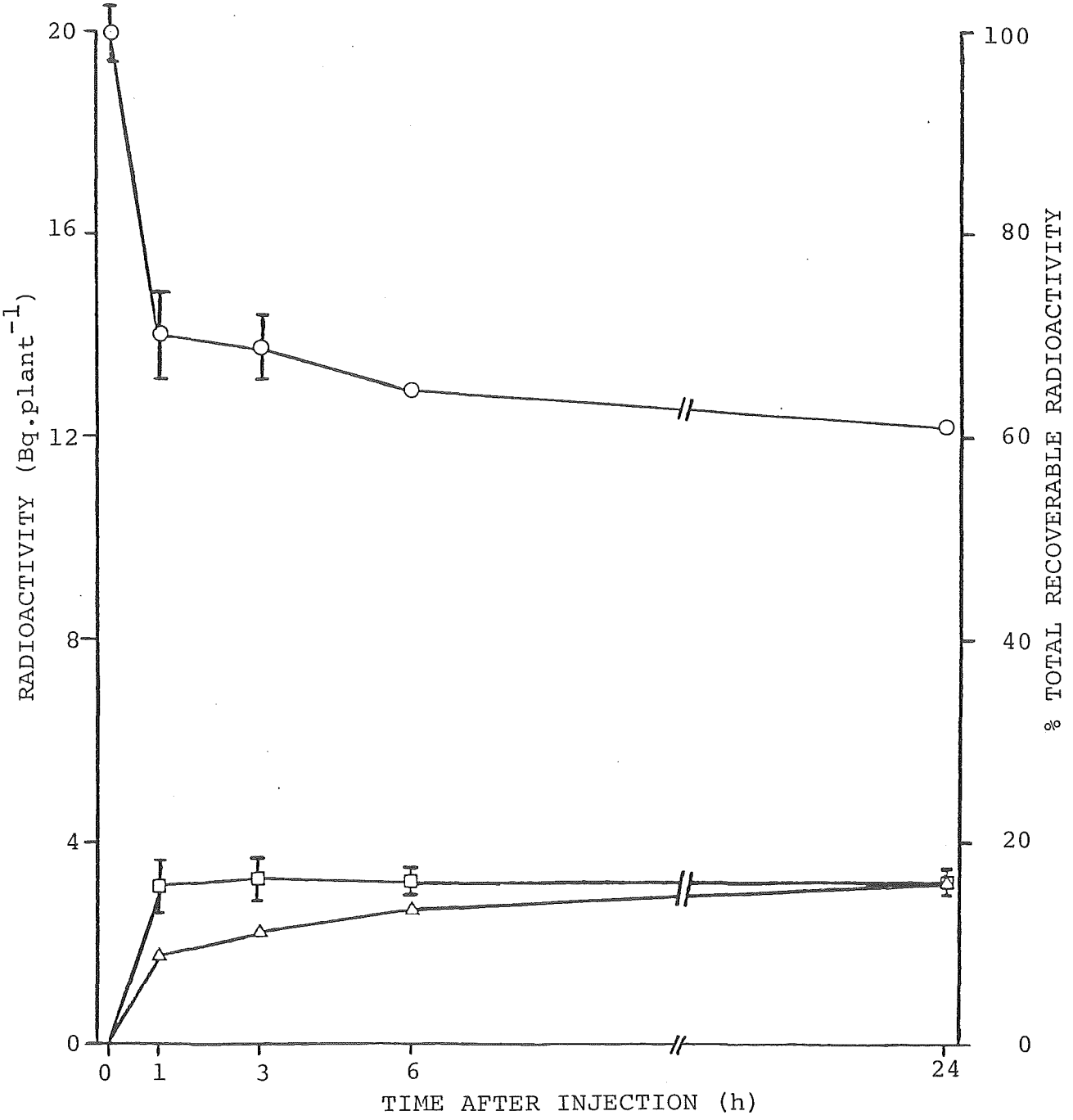

RADIOACTIVITY (Bq.plant^{-1})
% TOTAL RECOVERABLE RADIOACTIVITY
TIME AFTER INJECTION (h)
0
4
8
12
16
20
0
20
40
60
80
100
0
1
3
6
24

Figure 3.43: Time-course of distribution of radioactivity in seedlings following injection into the endosperm of 20 Bq IAA-^{14}C

For grains and roots, n = 10 (where no standard error is shown, the error term is less than or equal to the symbol used to designate the mean).

For shoot n = 1.

O Grain

Δ Shoot

□ Roots

was rapid initially, but which declined progressively as the incubation time after IAA application increased. In the 200 Bq experiment (Fig. 3.43), the bulk of the transport from the grain occurred in the first hour after injection. In the 2 kBq and 200 Bq experiments, transport was mostly in the first 3 h.

With all 3 series of experiments, the roots gained radioactivity more rapidly than the shoot. However, accumulation of label in the roots ceased after 1 h in the 20 Bq experiment, and 3 h in the 2 kBq and 200 Bq experiments, whereas the shoot continued to gain radioactivity slowly, throughout the 24 h experimental period. By 6 h after injection, the total amount of radioactivity in the shoot in the 2 kBq and 200 Bq experiments, exceeded that in the roots (Figs. 3.41 and 3.42), and by the end of the 20 Bq experiment, the amounts in the shoot and root were approximately equivalent (Fig. 3.43), at 15.9 and 16.0% of the total radioactivity in the seedling, respectively. After the same period, the shoot and roots of seedlings in the 2 kBq experiment contained 13.9% and 12.6% of the total recoverable radioactivity, while in the 200 Bq experiment these figures were 20.1 and 15.1%, respectively.

Not all the applied radioactivity could be recovered from the seedlings. Some was recoverable from the blotting paper strips that the plants grew on, in the water reservoir and the KOH trap in the bottom of each test tube. Table 3.15 shows the amount of radioactivity in each of these fractions over time, for each experimental series. In all cases, the majority of radioactivity occurring outside the plant was in the paper strips. The amount remained approximately constant with time, suggesting the most likely source of the label on the paper was leakage from the grain as the syringe was withdrawn, rather than from exudation from the roots, where an increase with time would have been expected. Similarly, a constant amount of radioactivity was present in the water reservoir that moistened the paper strips, but in this case the quantity involved was extremely low. A steady increase in the amount of radioactivity in the KOH trap was observed as the duration of the experiments increased.

By the end of the experimental period, these combined fractions accounted for almost 10% of the radioactivity in the

Table 3.15: Proportion of total radioactivity recovered outside the plant at various times following injection of 2 kBq, 200 Bq or 20 Bq IAA-^{14}C

Amount of IAA-^{14}C injected	Fraction	% of total recoverable radioactivity			
		time after injection (h)			
		1	3	6	24
2 kBq	Blotting paper	3.09±0.72	2.79±0.81	3.87±0.61	3.75±0.78
	Water reservoir	0.17±0.03	0.17±0.03	0.24±0.04	0.19±0.03
	KOH trap	0.31±0.04	0.87±0.38	2.02±0.28	2.90±0.45
	Total	3.57±0.79	3.87±1.22	6.13±0.93	6.84±1.26
200 Bq	Blotting paper	4.14±1.03	4.99±0.60	3.50±0.61	5.58±1.39
	Water reservoir	0.22±0.03	0.30±0.05	0.29±0.05	0.32±0.06
	KOH trap	0.52±0.05	0.77±0.07	1.07±0.31	4.09±1.52
	Total	4.88±1.11	6.06±0.72	4.86±0.97	9.99±1.97
20 Bq	Blotting paper	3.63±0.21	2.95±0.24	3.47±0.30	3.15±0.18
	Water reservoir	0.45±0.03	0.54±0.04	0.41±0.05	0.47±0.04
	KOH trap	0.48±0.08	0.76±0.09	1.10±0.13	1.98±0.39
	Total	4.56±0.32	4.25±0.37	4.98±0.48	5.60±0.61

200 Bq experiment, and 6.8 and 5.6% in the 2 kBq and 20 Bq experiments, respectively. These differences would partly account for the greater loss of radioactivity from the grain that was observed in the 200 Bq experiment. In addition, the figures on the amount of radioactivity remaining in the grain, and the amount transported into the shoot and roots, were affected by whether the scutellum was extracted with the grain or with the shoot. In the 2 kBq experiments, which were the first to be performed, some shoots became detached at the base, leaving the scutellum adhering to the endosperm. However, in the 200 Bq and 20 Bq series, particular care was taken to avoid this.

So that data were more comparable between experiments, radioactivity in the scutellum (estimated as the difference between the amount of label in the MD and MC segments of the mesocotyl - Figs 3.48 - 3.50, below) was subtracted from the total amount of radioactivity found in the shoot at the end of each experiment. This enabled the proportion of the applied radioactivity present in the shoot alone, to be calculated. Thus, the proportion of radioactivity transported from the grain into the shoot in 24 h was 11.7%, 15.0%, and 11.8% in the 2 kBq, 200 Bq and 20 Bq experiments, respectively.

3.4.1.2 Distribution of radioactivity in the shoot

(i) *Whole shoot.* During the course of each time-course experiment, extension growth of both the coleoptile and the mesocotyl proceeded in a linear fashion (Fig. 3.44). These data were derived from separate sets of plants, which were selected for length in the usual manner (section 2.3), and harvested at intervals thereafter. The fresh and dry weights of the different parts of the shoot were also determined (Table 3.16). Both these parameters correlated closely with the increase in segment length.

Figs 3.45, 3.46 and 3.47 show the total amount of label in the shoot at each harvest time following injection of 2 kBq, 200 Bq and 20 Bq IAA-^{14}C, as a function of the

Figure 3.44: Extension growth of the shoot during the experimental period

Values represent the mean ± S.E. for 21 seedlings. Where no S.E. is shown the error term is less than or equal to the size of the symbol used to designate the mean.

○ Total shoot length
△ Mesocotyl length
□ Coleoptile length

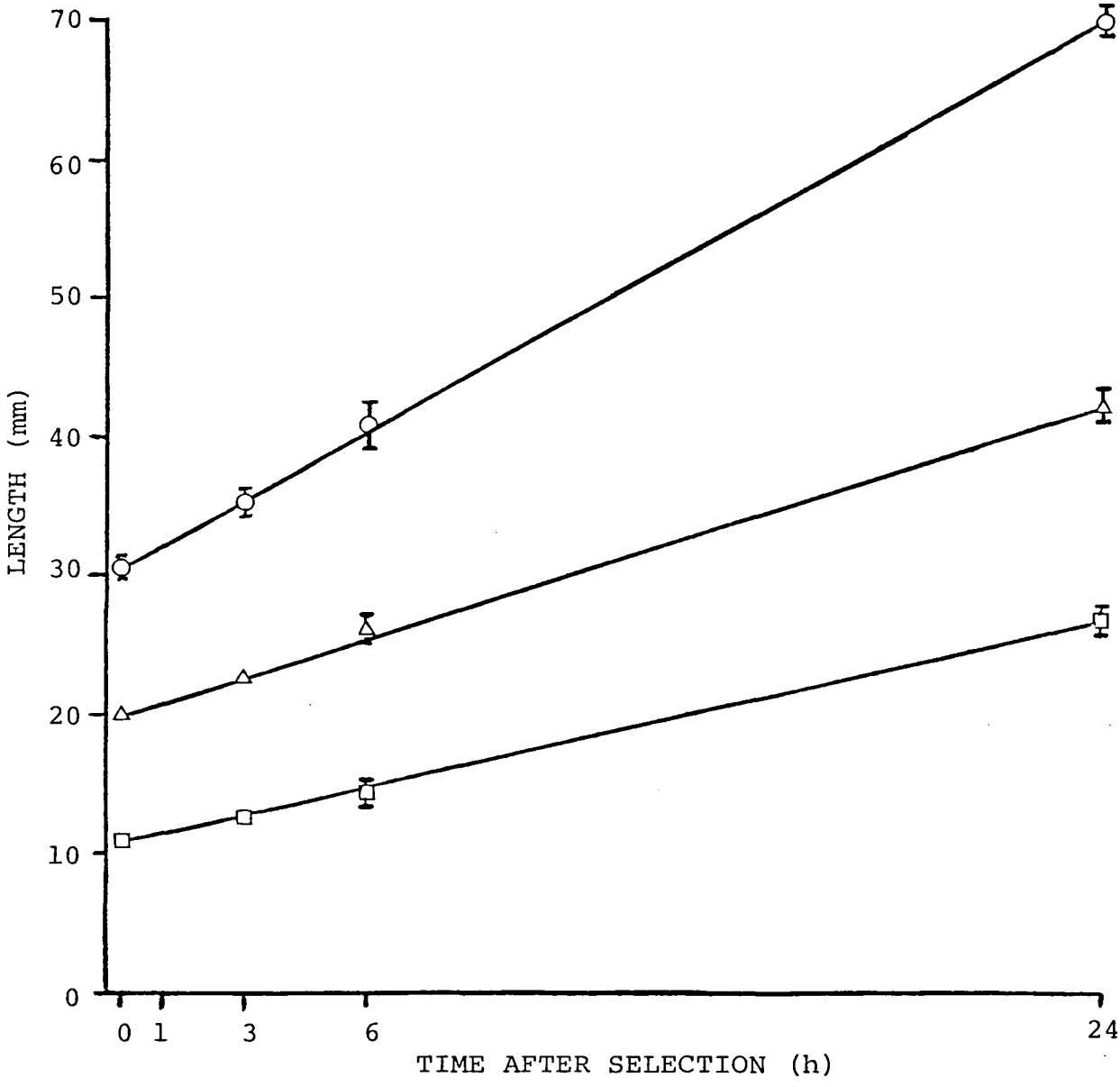

70
60
50
40
30
20
10
0
LENGTH (mm)
0 1 3 6 24
TIME AFTER SELECTION (h)

Table 3.16: Fresh and dry weights of shoot segments

Plants were selected for length at age 63 h and harvested at intervals thereafter. The values shown are the average weights per segment, computed from the total weight of 30 replicate segments. For the position of the segments, and the meaning of the abbreviations, see Fig. 2.1 (Materials and Methods).

Shoot segment	Fresh weight.segment^{-1} (mg)				Dry weight.segment^{-1} (mg)			
	1h	3h	6h	24h	1h	3h	6h	24h
MD	4.6	4.9	5.2	8.2	0.41	0.43	0.46	0.64
MC	3.9	4.2	4.6	7.9	0.23	0.25	0.27	0.47
MB	4.3	4.6	5.2	8.2	0.23	0.25	0.28	0.48
MA	4.6	5.1	5.6	8.8	0.27	0.30	0.33	0.53
N	3.6	3.7	3.7	4.0	0.37	0.37	0.38	0.42
L	2.7	2.9	3.4	6.1	0.50	0.55	0.64	1.12
C	6.8	7.7	9.3	17.5	0.50	0.57	0.69	1.35
T	1.5	1.5	1.4	1.5	0.13	0.14	0.14	0.13

Figure 3.45: Time-course of radioactivity in the shoot as a function of shoot fresh weight following injection into the endosperm of 2 kBq IAA-^{14}C

O Total shoot

Δ Shoot, excluding the scutellum-containing quarter of the mesocotyl

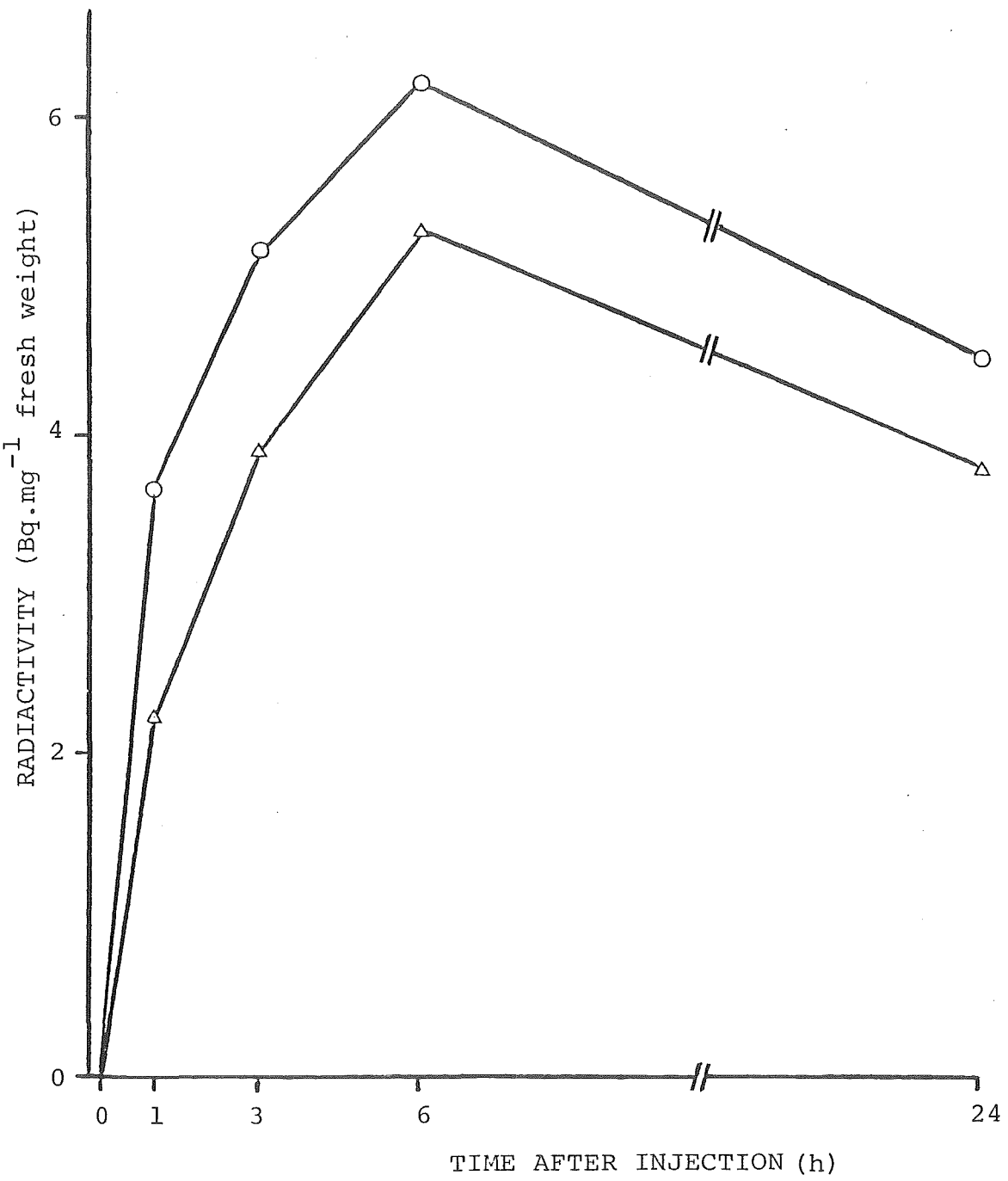

RADIACTIVITY (Bq.mg^{-1} fresh weight)
0
2
4
6
0
1
3
6
24
TIME AFTER INJECTION (h)

Figure 3.46: Time-course of radioactivity in the shoot as a function of shoot fresh weight following injection into the endosperm of 200 Bq IAA-^{14}C

○ Total shoot

△ Shoot, excluding the scutellum-containing quarter of the mesocotyl

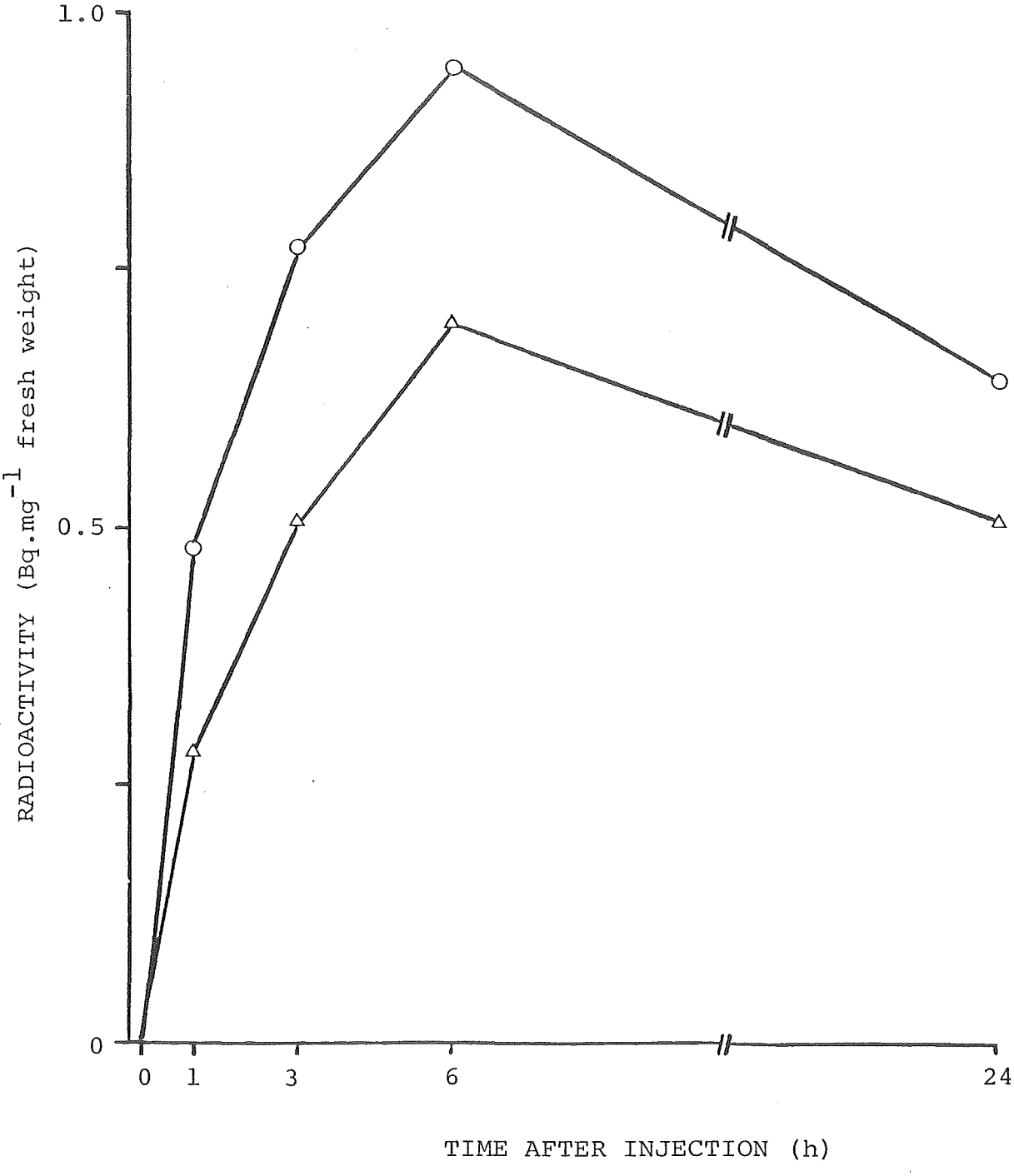
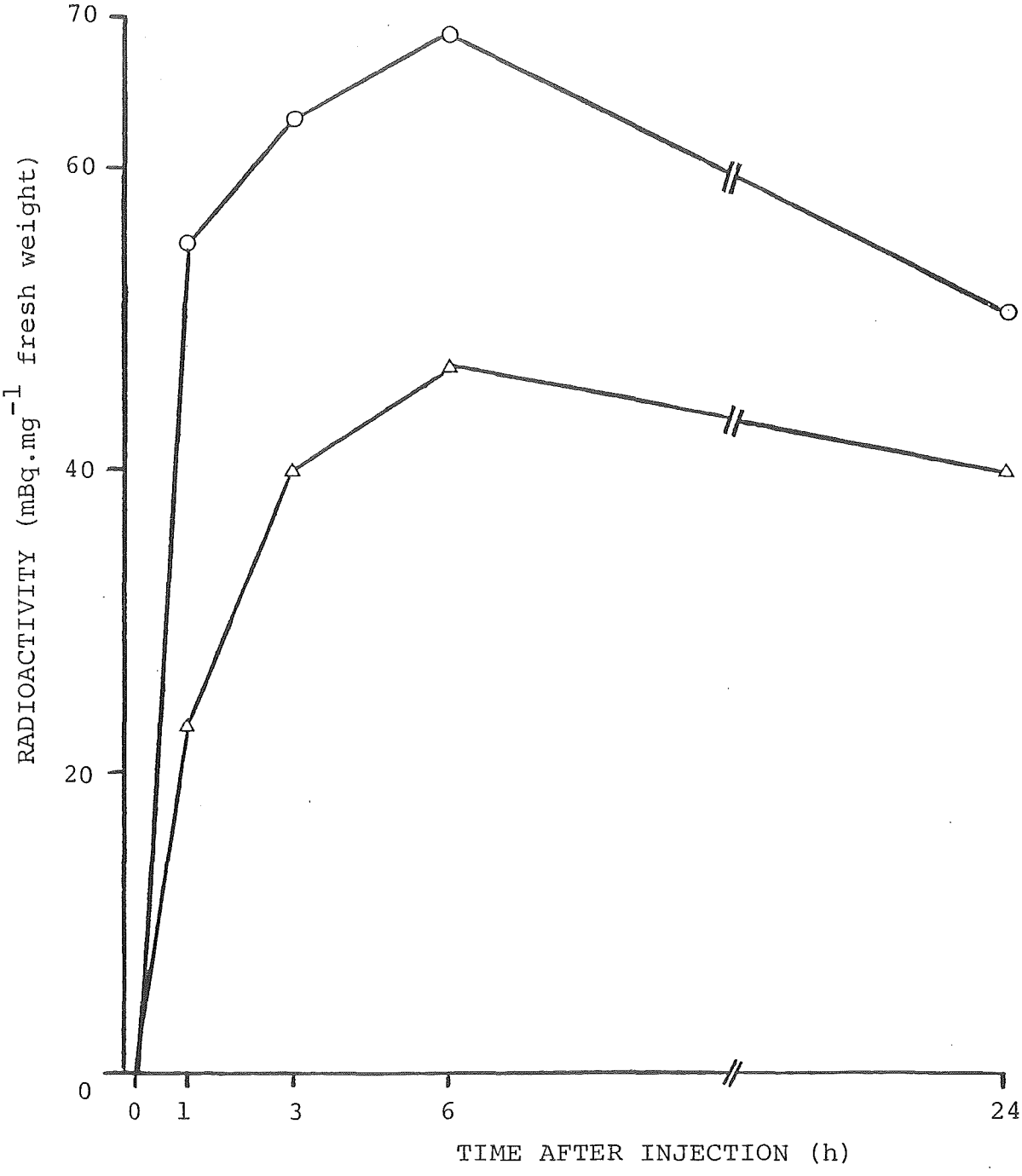

1.0
0.5
0
RADIOACTIVITY (Bq.mg^{-1} fresh weight)
0
1
3
6
24
TIME AFTER INJECTION (h)

Figure 3.47: Time-course of radioactivity in the shoot as a function of shoot fresh weight following injection into the endosperm of 20 Bq IAA-^{14}C

O Total shoot

Δ Shoot, excluding the scutellum-containing quarter of the mesocotyl


70
60
40
20
0
RADIOACTIVITY (mBq.mg^{-1} fresh weight)
0
1
3
6
24
TIME AFTER INJECTION (h)

total shoot fresh weight. To provide a better estimate of the radioactivity in the shoot tissue itself, the data were also calculated after deduction of the radioactivity in, and the fresh weight of, the scutellum part of the shoot (MD segment).

In the 2 kBq and 200 Bq experiments (Fig. 3.45 and 3.46), both estimates indicated rapid accumulation of radioactivity in the shoot, with a pronounced peak 6 h after IAA-^{14}C injection and a subsequent decline. So by the end of the 24 h experimental period, the average radioactivity levels of the tissue had fallen to near the levels occurring at the 3 h harvest time. The difference between the 'total shoot' curve and the curve for the shoot data calculated without the scutellum, was greater in the 200 Bq experiment, reflecting the larger quantities of radioactivity in the scutellum.

In the 20 Bq experiment, an essentially similar pattern was observed (Fig. 3.47), although the peak of radioactivity in the tissue 6 h after injection, was less pronounced than in the other two series. Nevertheless, in all 3 cases, the concentration of radioactivity in the shoot (excluding the scutellum) at the end of 24 h incubation, relative to the amount injected, was very similar (3.84 Bq.mg^{-1} fresh weight in the 2 kBq experiment, and 0.507 Bq.mg^{-1} and 40 mBq.mg^{-1} for the 200 Bq and 20 Bq experiments respectively).

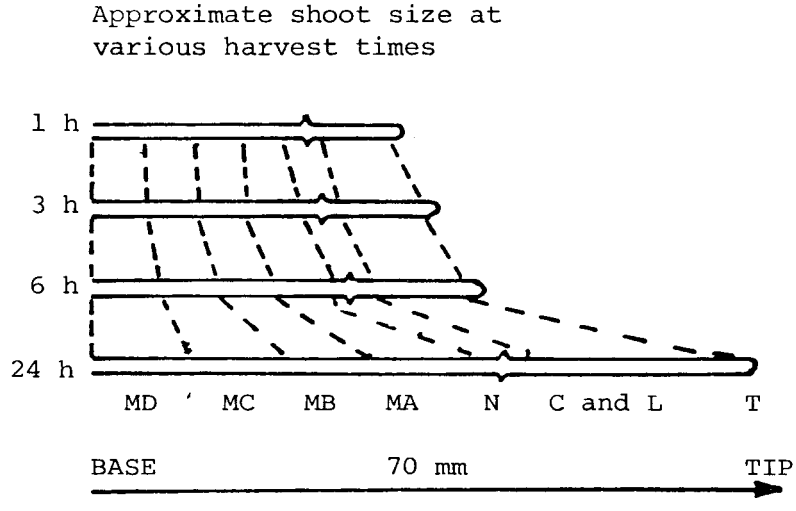
A basically similar pattern was observed if the data were expressed as a function of shoot dry weight.

(ii) *Total radioactivity per segment.* The distribution of radioactivity within the various sections of the developing shoot, following injection of 2 kBq, 200 Bq and 20 Bq of IAA-^{14}C, is shown in Figs. 3.48, 3.49 and 3.50. In all 3 series, the resultant distribution of radioactivity throughout the shoot was very similar.

Radioactivity transported out of the grain became distributed rapidly throughout the length of the shoot

Figure 3.48: Time-course of distribution of radioactivity within the developing shoot after injection into the endosperm of 2 kBq IAA-^{14}C

Where no standard error is shown, the error term is less than 0.5 Bq.



T = Coleoptile Tip

C = Coleoptile

L = Primary Leaves (inside coleoptile)

N = Coleoptilar Node

MA, MB, MC, MD = Quarter lengths of Mesocotyl

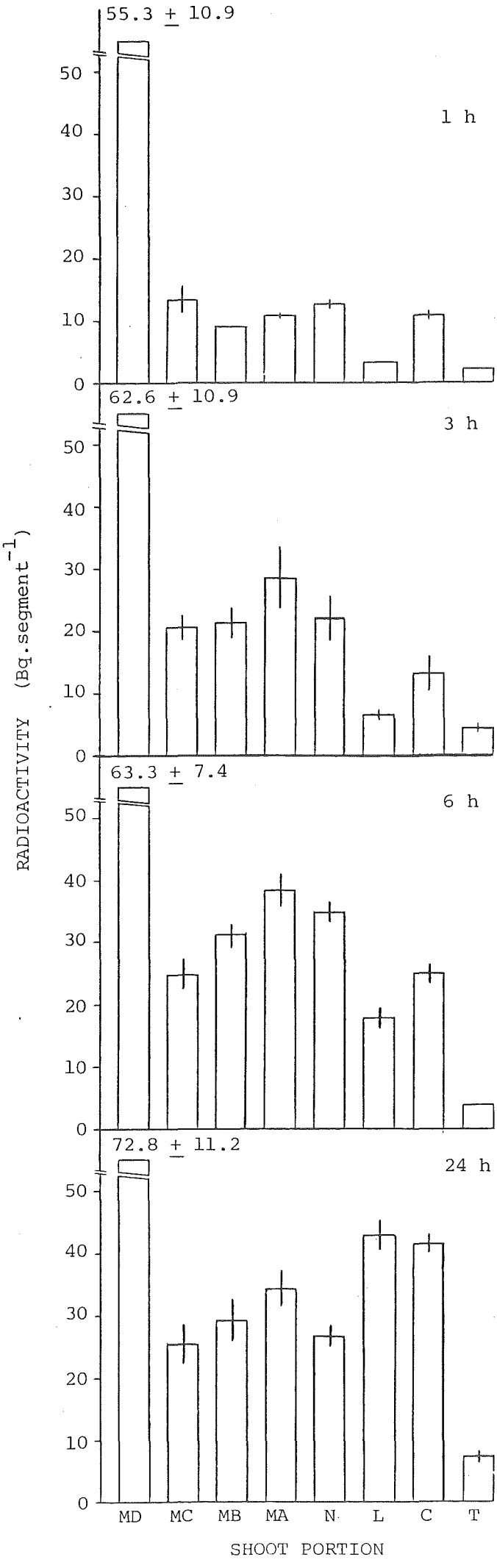
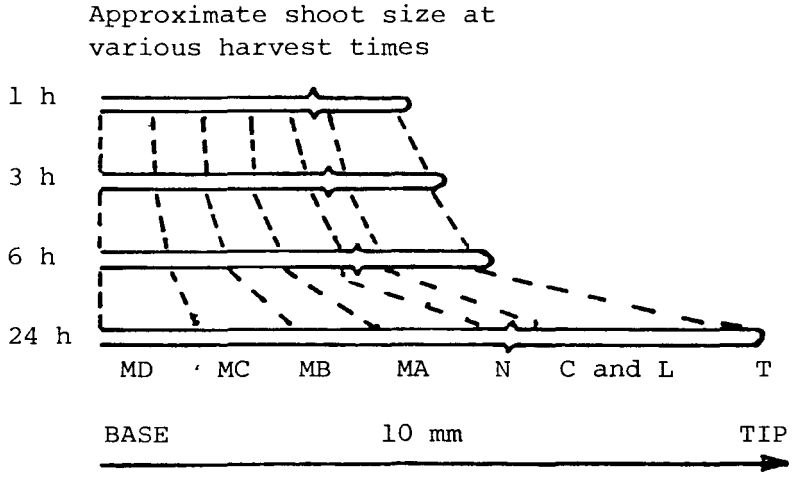

55.3 ± 10.9
1 h
62.6 ± 10.9
3 h
63.3 ± 7.4
6 h
72.8 ± 11.2
24 h
0
10
20
30
40
50
RADIOACTIVITY (Bq.segment^{-1})
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

Figure 3.49: Time-course of distribution of radioactivity within the developing shoot after injection into the endosperm of 200 Bq IAA-^{14}C

Where no standard error is shown, the error term is less than 0.05 Bq.



T = Coleoptile Tip

C = Coleoptile

L = Primary Leaves (inside coleoptile)

N = Coleoptilar Node

MA, MB, MC, MD = Quarter lengths of Mesocotyl

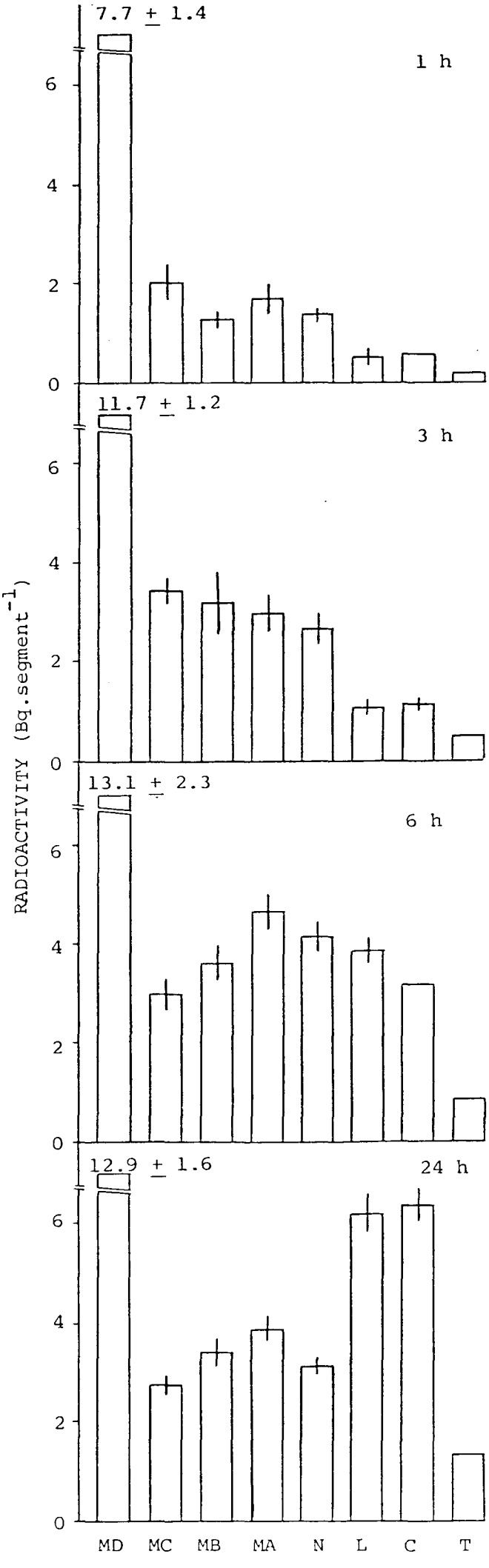
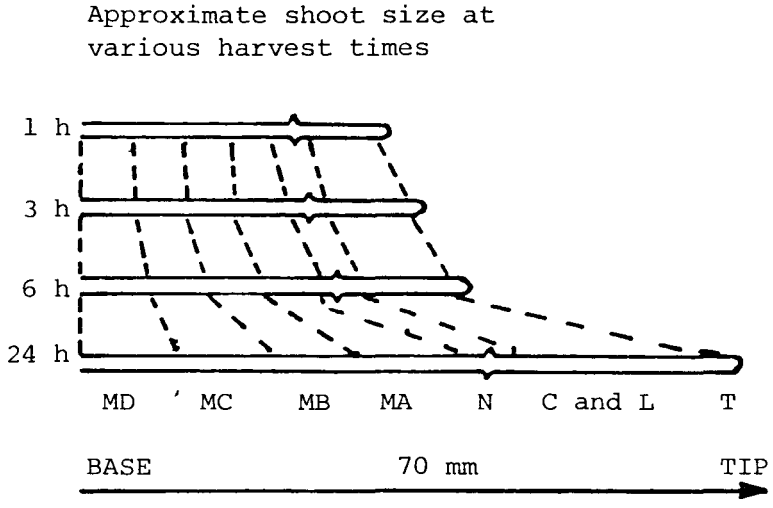

7.7 ± 1.4
1 h
11.7 ± 1.2
3 h
13.1 ± 2.3
6 h
12.9 ± 1.6
24 h
RADIOACTIVITY (Bq.segment^{-1})
0
2
4
6
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

Figure 3.50: Time-course of distribution of radioactivity within the developing shoot after injection into the endosperm of 20 Bq IAA-^{14}C



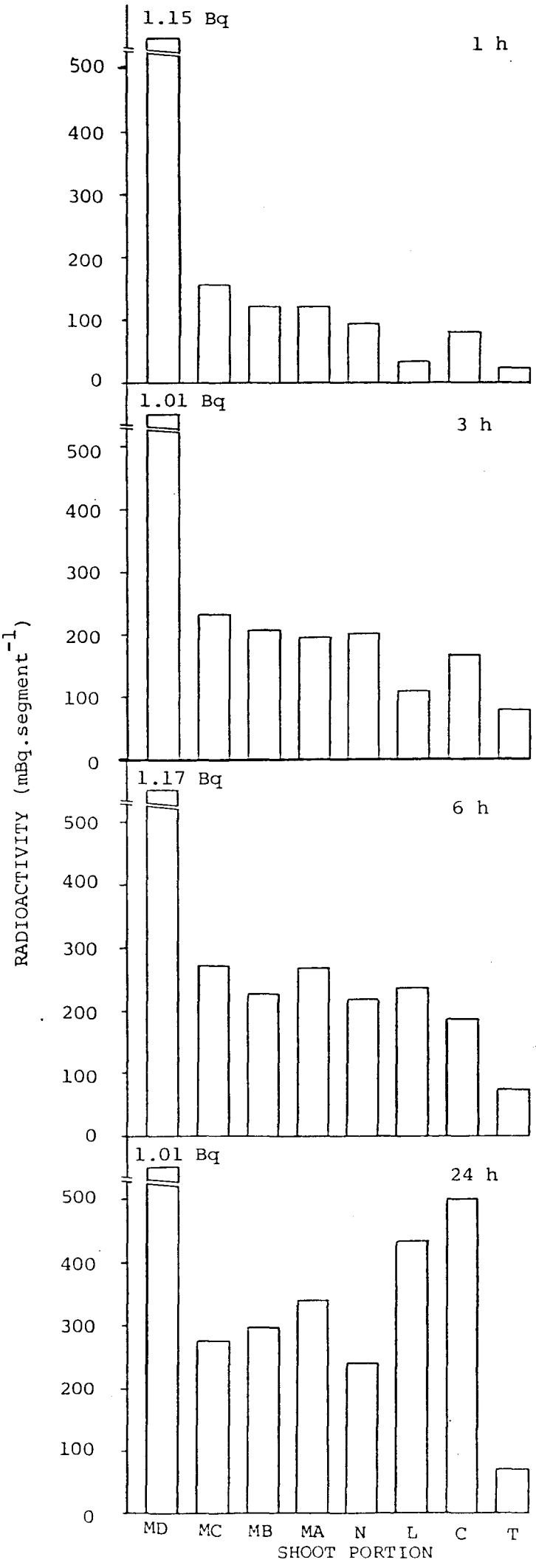
T = Coleoptile Tip

C = Coleoptile

L = Primary Leaves (inside coleoptile)

N = Coleoptilar Node

MA, MB, MC, MD = Quarter lengths of mesocotyl


1.15 Bq
1 h
1.01 Bq
3 h
1.17 Bq
6 h
1.01 Bq
24 h
RADIOACTIVITY (mBq.segment^{-1})
0
100
200
300
400
500
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

(preliminary experiments showed activity was detectable at the tip of the coleoptile 5 min after injection of 2 kBq of IAA-^{14}C into the endosperm). One hour after injection, significant amounts of radioactivity were present at the coleoptile tip (T), and in the coleoptile (C) and enclosed first leaves (L) (Figs 3.48, 3.49 and 3.50). However, in all 3 experiments, over 75% of the radioactivity was contained in the mesocotyl. Much of this was in the basal, scutellum-containing segment (MD). A later experiment, in which the lower quarter of the mesocotyl and the scutellum were analysed separately, showed that the bulk of the label was contained in the scutellum, and that the level of the radioactivity in the mesocotyl segment alone, was similar to the level observed in the neighbouring tissue.

In each experimental series, the relative distribution of radioactivity between the coleoptile and mesocotyl was preserved as additional radioactivity was transported into the shoot, in the 1 to 3 h interval after IAA-^{14}C injection. Radioactivity continued to accumulate in all segments up to the 6 h harvest, but there was a noticeable increase in the proportion of label contained in the coleoptile (including the tip and first leaves), relative to the mesocotyl. In particular, the percentage of radioactivity in the primary leaves approximately doubled between the 3 h and 6 h harvests (from 3.7 - 4.8% to 7.4 - 8.9%).

The coleoptile, coleoptile tip and primary leaves continued to gain radioactivity in the following 18 h. By the end of the experimental period, the coleoptile and first leaves individually contained the highest proportion of radioactivity of any shoot portion, with the exception of the scutellum-containing segment of the mesocotyl. In this period, in the 2 kBq and 200 Bq experiments (Figs 3.48 and 3.49), the coleoptilar node (N) and the upper half of the mesocotyl (MA, MB) lost radioactivity, presumably to the coleoptile and first leaves. In the 20 Bq experiment, the amount of label in the upper three-quarters of the mesocotyl and coleoptilar node remained approximately constant

in the 6 to 24 h period after injection, but there was a decrease in radioactivity in the scutellum-containing segment of the mesocotyl (Fig. 3.50).

At the end of the 2 kBq experiment, the coleoptile tip and first leaves contained 32.7% of the total radioactivity in the shoot, the coleoptilar node 9.5%, and the mesocotyl 57.8%. In the 200 Bq and 20 Bq experiments, these proportions were 35.3:7.8:56.9 and 31.8:7.8:60.6, respectively.

(iii) *Radioactivity per segment on a fresh weight basis.* For each IAA-^{14}C application rate, the amount of radioactivity in each part of the shoot, at the various harvest times was calculated as a function of the tissue fresh weight (see Table 3.16, above). These data are shown in Figs 3.51, 3.52 and 3.53. In the early stages of each experiment, radioactivity levels were generally highest in the mesocotyl and the coleoptilar node. These increased to a peak 6 h after IAA-^{14}C injection, and then declined by 24 h to between the levels that occurred at the 1 and 3 h harvests.

The levels of radioactivity in the coleoptile remained approximately constant after an initial increase and, except at the final harvest when levels were almost equal, were much less than in the mesocotyl tissues. Within the coleoptile, radioactivity tended to be concentrated at the tip and, at most harvest times, the levels were at least double those in the rest of the coleoptile. In the 2 kBq and 20 Bq experiments (Figs. 3.51 and 3.53), radioactivity accumulated in the coleoptile tip and then the levels remained constant, whereas in the 200 Bq experiment (Fig. 3.52), label continued to accumulate at the tip throughout the experiment. In all cases, by the final harvest, the radioactivity levels in the coleoptile tip exceeded those in the mesocotyl (with the exception of the scutellum-containing portion). Both the coleoptilar node segment and the primary leaves also contained particularly high amounts of radioactivity, relative to their fresh weight.

Although not shown here, when the data were expressed on a dry weight basis, the radioactivity levels in the

Figure 3.51: Time-course of radioactivity in each shoot segment, as a function of segment fresh weight, following injection into the endosperm of 2 kBq IAA-^{14}C

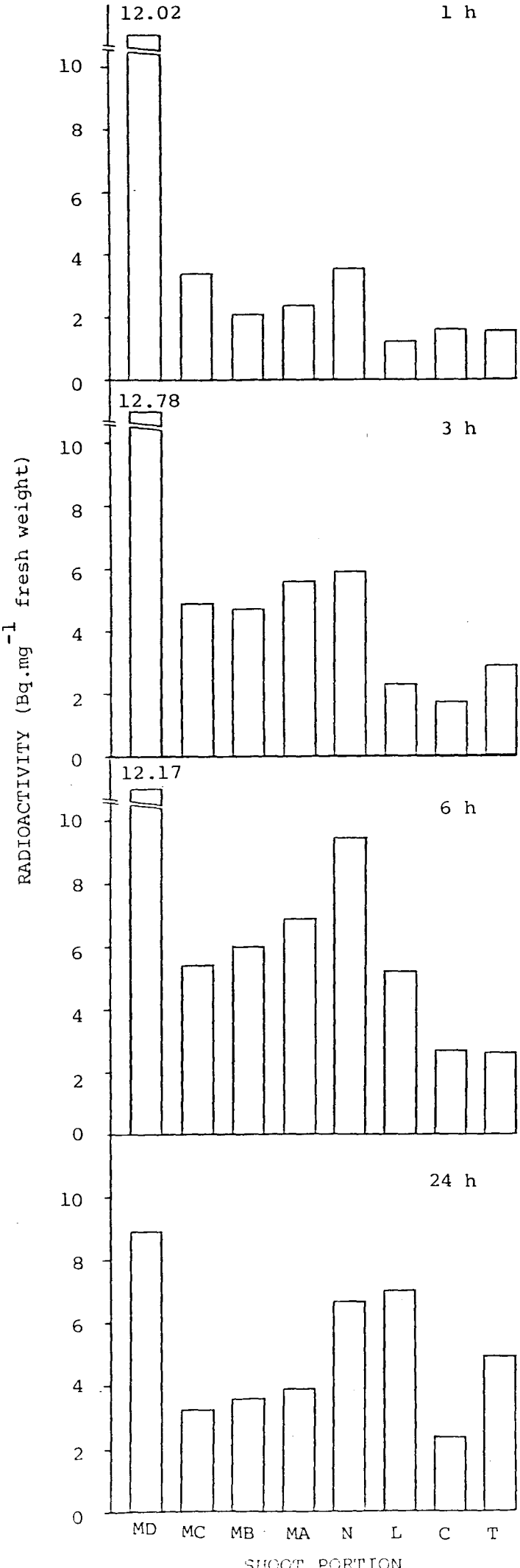

12.02
1 h
12.78
3 h
12.17
6 h
24 h
RADIOACTIVITY (Bq.mg-1 fresh weight)
0
2
4
6
8
10
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

Figure 3.52: Time-course of radioactivity in each shoot segment, as a function of segment fresh weight, following injection into the endosperm of 200 Bq IAA-^{14}C

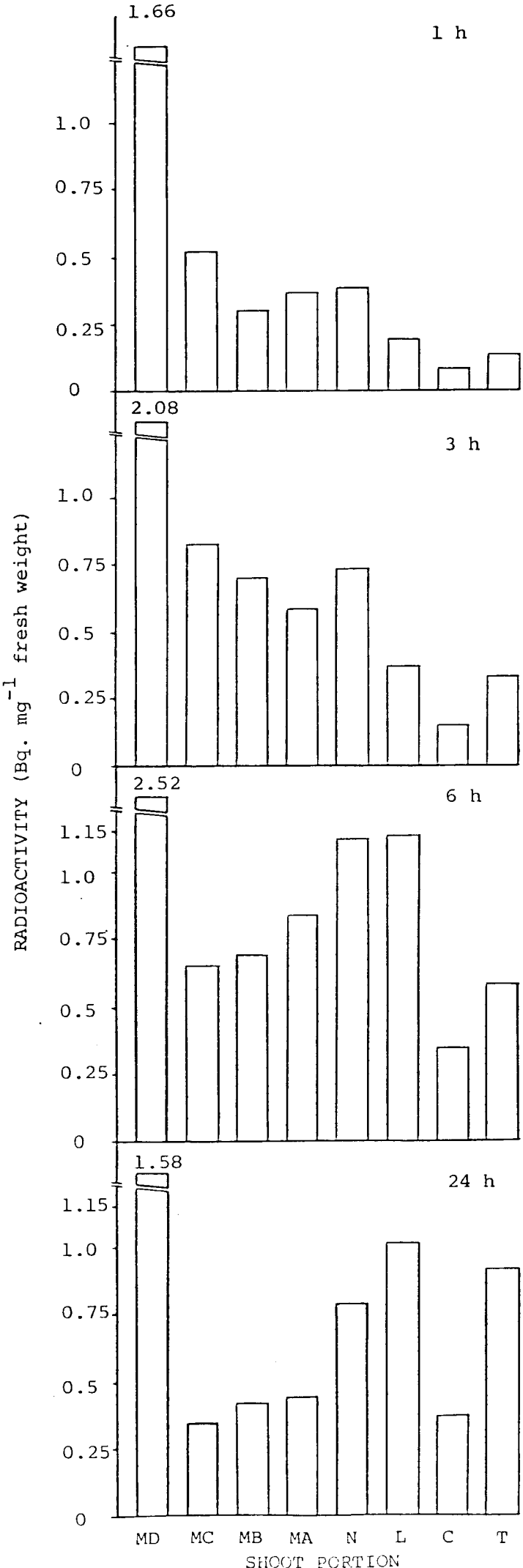
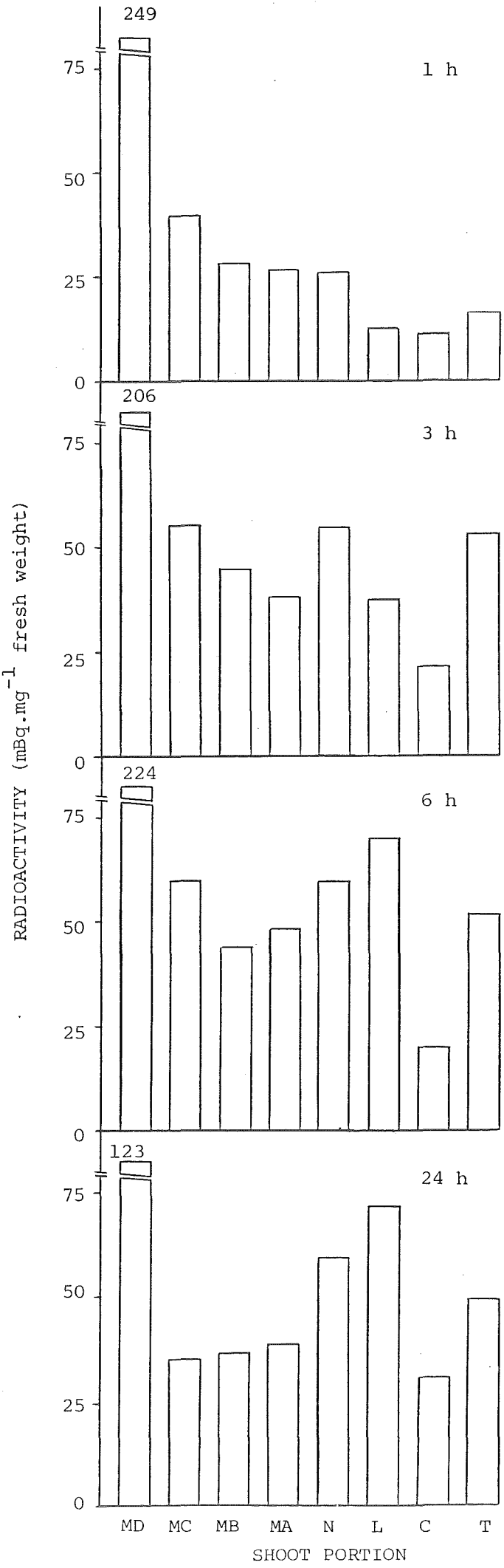

1.66
1 h
1.0
0.75
0.5
0.25
0
2.08
3 h
1.0
0.75
0.5
0.25
0
2.52
6 h
1.15
1.0
0.75
0.5
0.25
0
1.58
24 h
1.15
1.0
0.75
0.5
0.25
0
RADIOACTIVITY (Bq. mg^{-1} fresh weight)
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

Figure 3.53: Time-course of radioactivity in each shoot segment, as a function of segment fresh weight, following injection into the endosperm of 20 Bq IAA-^{14}C


249
1 h
206
3 h
224
6 h
123
24 h
75
50
25
0
RADIOACTIVITY (mBq.mg^{-1} fresh weight)
MD
MC
MB
MA
N
L
C
T
SHOOT PORTION

coleoptilar node and primary leaves were reduced considerably relative to the rest of the shoot, due to the comparatively high dry matter contents of these tissues (Table 3.16, above). Because of the low dry matter contents of the mesocotyl tissues these data were attenuated relative to the coleoptile tissues.

In an attempt to determine how translocation from the endosperm occurs, 0.2% aqueous acid fuschin dye (w/v) was supplied to 63h old dark-grown seedlings. This was done by inserting a pasteur pipette, whose end had been drawn out to a fine point, into the endosperm. A small amount of dye was placed in the pipette and the whole assembly was incubated in darkness at 24°C. Other plants were supplied with dye via the roots, which were cut to facilitate entry of the dye.

At intervals, plants were removed from the darkroom and transverse sections of the shoot cut. Dye supplied either to the roots or to the endosperm was found to accumulate at the coleoptile tip. In both cases, accumulation could be enhanced by increasing the rate of transpiration by placing the seedlings in front of a fan. Dye supplied to the endosperm was transported in the cortical vascular bundle in the mesocotyl, through the coleoptile bundles, to the coleoptile tip. Dye also moved progressively down the central stele towards the roots.

The reverse was true for dye supplied to the roots; i.e. dye moved up the central stele en route to the coleoptile tip, and was transported basipetally in the cortical vascular bundle. In shoots placed with their cut bases in dye solutions, transport occurred in both the cortical and central vascular bundles.

3.4.1.3 IAA-^{14}C metabolism.

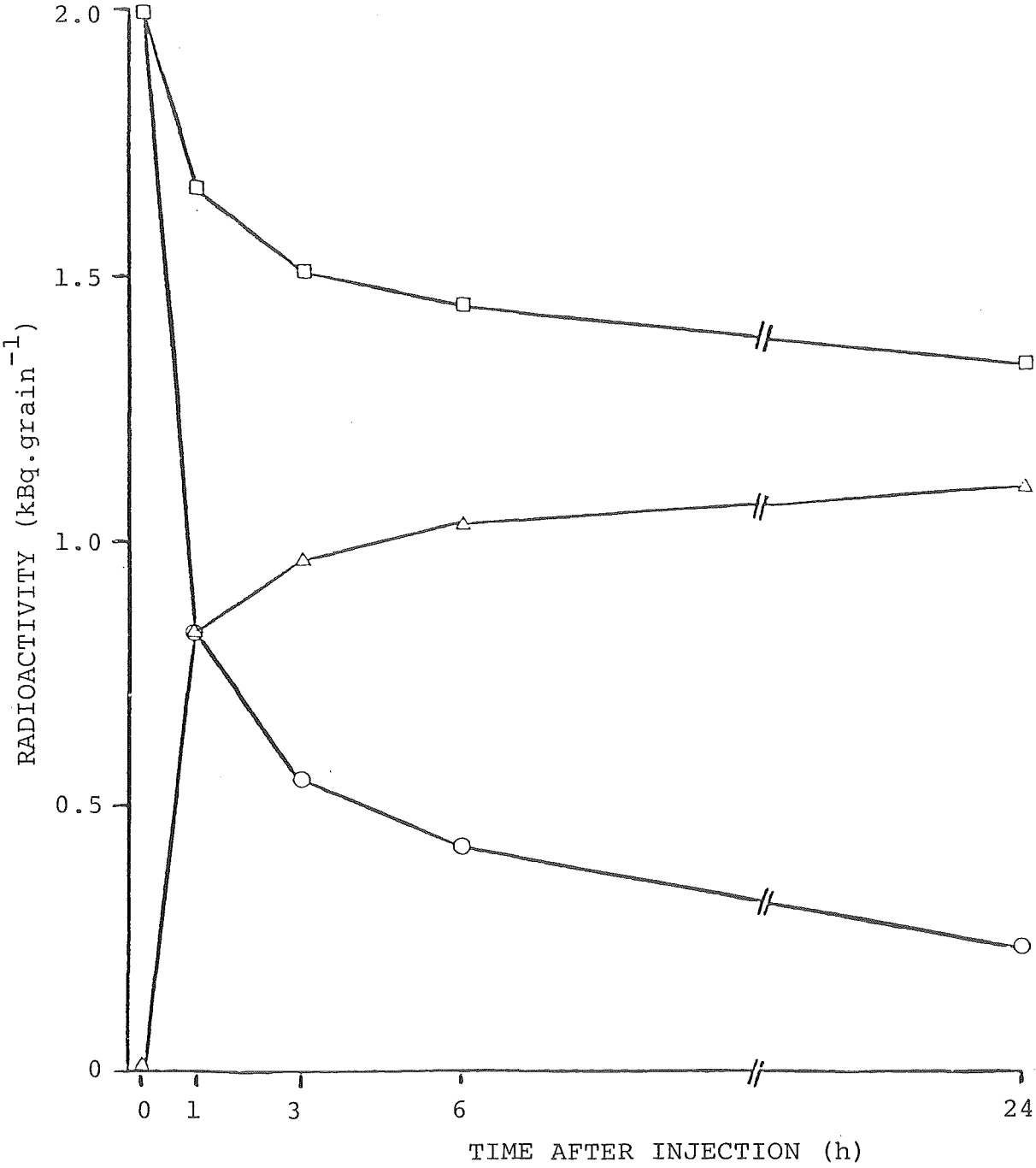
(i) *Grain.* Only in the 2 kBq experiment was a full time-course study of IAA-^{14}C metabolism, following injection into the endosperm, carried out. Fig. 3.54 shows that metabolism of IAA-^{14}C in the grain was extremely rapid in

Figure 3.54: Time-course of levels of IAA-^{14}C and labelled metabolites in the grain following injection of 2 kBq IAA-^{14}C.

□ Total radioactivity

○ IAA-^{14}C

△ Metabolites


2.0
1.5
1.0
0.5
0
RADIOACTIVITY (kBq.grain^{-1})
0
1
3
6
24
TIME AFTER INJECTION (h)

the first hour after injection, but that the rate slowed progressively thereafter. Over 41% of the applied IAA was metabolised within an hour of injection, but in the following 23 h period, this proportion increased only slightly to 54%.

The export of radioactivity from the grain, and the slow but continued metabolism of IAA-^{14}C, meant that the proportion of label in the grain comprised of free IAA, fell steadily throughout the experiment, from 36.4% after 3 h, to 29.1% after 6 h, and 17.6% at the end of the 24 h experimental period. These proportions represented 27.5%, 21.3% and 11.8% of the applied IAA-^{14}C, respectively.

In the 200 Bq and 20 Bq experiments, IAA-^{14}C metabolism was determined only at the 6 h harvest. At this stage, in the 200 Bq experiment, 20.9% of the radioactivity remaining in the grain was in the form of free IAA. For the 20 Bq experiment this figure was 18.7%. As a proportion of the amount of IAA-^{14}C originally applied, this represented 13.3% and 12.9%, respectively, for the 200 and 20 Bq injections.

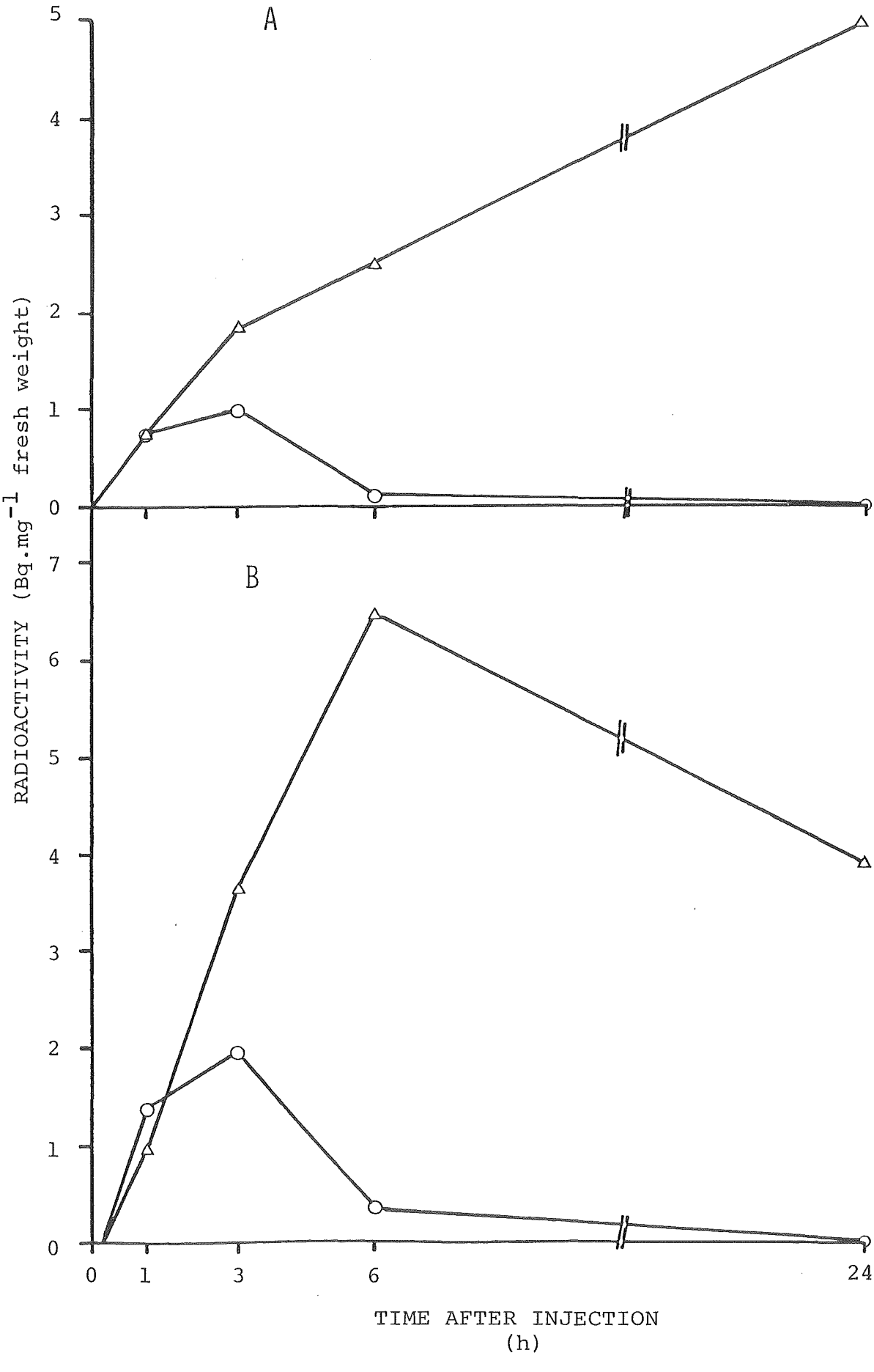
(ii) *Shoot*. The nature of the radioactive compounds in the apical segment of the mesocotyl and the coleoptile tip were determined. As with the grain, the full time-course was studied only in the 2 kBq experiment (Fig. 3.55).

Initially both free and metabolised forms of IAA-^{14}C were present in the coleoptile tip and upper mesocotyl, but the free IAA rapidly disappeared, indicating that metabolism of IAA-^{14}C was occurring in the shoot. One hour after injection, the coleoptile tip and upper mesocotyl segment each contained approximately equal proportions of IAA-^{14}C and IAA-^{14}C metabolites. The IAA levels peaked at the 3 h harvest and then declined. So by 6 h after injection, free IAA comprised only about 5% of the total radioactivity in both the upper segment of the mesocotyl, and in the coleoptile tip. By the end of the 24 h experimental period, no IAA-^{14}C was detectable in either tissue. In the coleoptile tip, the level of labelled metabolites increased steadily throughout the experiment, while in the mesocotyl segment these rose to a peak after 6 h, and then declined to near the 3 h level.

Figure 3.55: Time-course of levels of IAA-^{14}C and labelled metabolites in (A) the coleoptile and (B) the apical quarter of the mesocotyl following injection into the endosperm of 2 kBq IAA-^{14}C

○ IAA-^{14}C

△ Metabolites


A
B
RADIOACTIVITY (Bq.mg^{-1} fresh weight)
0
1
2
3
4
5
6
7
0
1
3
6
24
TIME AFTER INJECTION
(h)

The nature of the radioactive compounds entering the shoot was investigated by analysing the radioactivity in the scutellum 6 h after IAA-^{14}C injection, using TLC. It was found that less than 2% of the total radioactivity in the scutellum consisted of free IAA-^{14}C.

The proportions of free and metabolite IAA-^{14}C extractable from the coleoptile tip and upper mesocotyl, 6 h after injection of 200 Bq and 20 Bq IAA-^{14}C, are shown in Table 3.17. In the 200 Bq experiment, free IAA comprised 5.4% of the radioactivity in the coleoptile tip and 6.1% in the mesocotyl. This was similar to the proportions present at the same stage following injection of 2 kBq IAA-^{14}C.

No IAA-^{14}C was detectable in either the coleoptile tip or the mesocotyl segment 6 h after injection of 20 Bq into the endosperm (Table 3.17).

Table 3.17: Proportions of IAA-^{14}C and labelled metabolites in the radioactivity extractable from the coleoptile tip, and apical segment of the mesocotyl, 6 h after injection of 200 Bq or 20 Bq of IAA-^{14}C

Fraction	Amount of IAA-^{14}C injected			
	200 Bq		20 Bq	
	Coleoptile tip	Mesocotyl	Coleoptile tip	Mesocotyl
IAA-^{14}C%	5.4	6.1	0	0
Metabolites %	94.6	93.9	100	100

3.4.2 Effect of light treatment on transport and metabolism of endosperm-applied IAA-^{14}C

Seedlings injected with 20 Bq IAA-^{14}C were given either a 10 s exposure to red light or maintained in darkness, and the amount and form of the radioactive compounds

in the shoot and grains determined after a further 6 h. Two separate experiments were performed (section 2.5.3).

3.4.2.1 Transport into the shoot

In both experiments, light treatment resulted in slightly less of the applied radioactivity being transported into the shoot (Table 3.18). However, this was not statistically significant [Student's t-test, $P < 0.05$]. Within the shoot, the effect of light on the distribution of radioactivity was highly significant in both experiments (Chi square 'goodness of fit', $P < 0.001$, in both cases). The difference between the distribution of radioactivity within the dark-grown plants in the duplicate experiments was also significant, but only at the 5% probability level (the experiments were, however, performed on different days). There was no significant difference between the light-treated plants in duplicate experiments (Chi square, $P < 0.01$).

Light-treated mesocotyls contained 17-19% less radioactivity than the dark controls, relative to the amount of label applied (Table 3.18). Such mesocotyls were also approximately 19% shorter, and weighed 16% less, than their dark-grown counterparts (Tables 3.19 and 3.20). On the other hand, there was a 34% increase in the proportion of radioactivity located in the primary leaves (Table 3.18), even though the weight and length of these was unaffected (Tables 3.19 and 3.20). The proportion of label in the coleoptile was not noticeably affected by light treatment.

3.4.2.2 Metabolism of IAA-^{14}C

The nature of the radioactive compounds in the coleoptile, mesocotyl and grain was determined in the light-treated and dark-grown plants, 6 h after injection, in the two replicate experiments (Table 3.21). Exposure of the seedlings to light at the time of injection had no apparent effect on the metabolism of IAA-^{14}C in the endosperm. In both the light-treated and dark-grown seedlings, no free IAA-^{14}C was detectable in either the coleoptile or the mesocotyl, and in both, the IAA-^{14}C

Table 3.18: Transport of radiactivity into the shoot of light-treated and dark-grown seedlings

Duplicate experiments were performed on different occasions (see section 2.5.3).

Plant Portion		DARK		LIGHT	
		mBq.portion^{-1}	% of total radio-activity in shoot and grain	mBq.portion^{-1}	% of total radio activity in shoot and grain
Expt. 1	coleoptile	495	2.99	479	2.94
	leaves	395	2.39	517	3.18
	mesocotyl	1697	10.26	1346	8.27
	shoot total	2587	15.64	2342	14.39
	grain	13,950		13,937	
grain and shoot total		16,537		16,279	
Expt. 2	coleoptile	440	2.80	428	2.66
	leaves	364	2.32	507	3.15
	mesocotyl	1511	9.63	1289	8.01
	shoot total	2315	14.75	2224	13.82
	grain	13,373		13,867	
grain and shoot total		15,688		16,091	

Table 3.19: Effect of light on shoot extension growth

Length of mesocotyl and coleoptile after receiving 10 s exposure to red light at age 63 h, compared to a dark control (determined from an experiment, separate from the one in Table 3.18 - see section 2.5.3).

Values are expressed ± standard error for 21 seedlings. The light vs dark effect was tested using Student's t-test (** = $P < 0.01$). (Lengths of coleoptile and mesocotyl at age 63 h were 10.8 ± 0.15 mm and 19.9 ± 0.36 mm, respectively).

Shoot portion	Length at age 69 h (mm)	
	Dark	Light
Coleoptile (and enclosed first leaves)	14.8 ± 0.47	14.7 ± 0.42 n.s.
mesocotyl	26.1 ± 0.52	21.3 ± 0.31 **

Table 3.20: Fresh weights of the shoots measured in Table 3.19

Shoot portion	Fresh weight at end of experiment (mg.21 shoots^{-1})	
	Dark	Light
coleoptile	270	272
primary leaves	74	72
mesocotyl	481	403

Table 3.21: Proportions of IAA-^{14}C and labelled metabolites in dark-grown and light-treated seedlings 6 h after injection of 20 Bq IAA-^{14}C

Plant Portion	Fraction	% of radioactivity			
		Experiment 1		Experiment 2	
		Dark	Light	Dark	Light
Coleoptile	IAA-^{14}C	0	0	0	0
	metabolites	100	100	100	100
mesocotyl	IAA-^{14}C	0	0	0	0
	metabolites	100	100	100	100
grain	IAA- C	18.6	19.5	21.2	20.0
	metabolites	81.4	80.5	78.8	80.0

metabolites were immobile when chromatographed in MEK/hexane. It is possible that light altered the rate of metabolism of IAA in the shoot, but this could not be ascertained from these experiments.

3.5 EFFECT OF GROWTH REGULATORS ON SEEDLING EXTENSION

The effects of exogenous applications of growth regulators on the extension growth of both, etiolated Avena seedlings, and those which had been briefly exposed to red-light, was studied. Applications were made to seedlings selected for uniformity of shoot length, 63 h after planting (section 2.3). The mean coleoptile, mesocotyl and root lengths of seedlings selected on this basis, determined over a number of experiments, were 10.8 $\pm$ 0.8 mm, 20.3 $\pm$ 1.3 mm and 54.3 $\pm$ 6.3 mm, respectively. The response of the seedlings to the growth regulators was measured after a further 24 h (i.e. at age 87 h). Where the interaction with light was studied, seedlings were exposed to red light for 10 s at the time of growth regulator application (section 2.2.2).

It was found that none of the growth regulator treatments affected the development of the primary roots, on either light-treated or dark-grown plants. Over all the experiments carried out, the range of mean root lengths was 61.1 mm to 70.7 mm; but in no instance, including following ABA application, was a statistically significant response to hormone treatment observed (split-plot analysis of variance). Therefore these results are not presented in detail below.

3.5.1 Indole-3-acetic acid

3.5.1.1 Injection into the endosperm

Injections of 2.5×10^{-3}, 2.5×10^{-4} and 2.5×10^{-5} M IAA were made (the amount of IAA applied when 2.5×10^{-4} M IAA was injected, 178 ng, was approximately equivalent to the amount applied in the 2 kBq IAA-^{14}C injection experiments above).

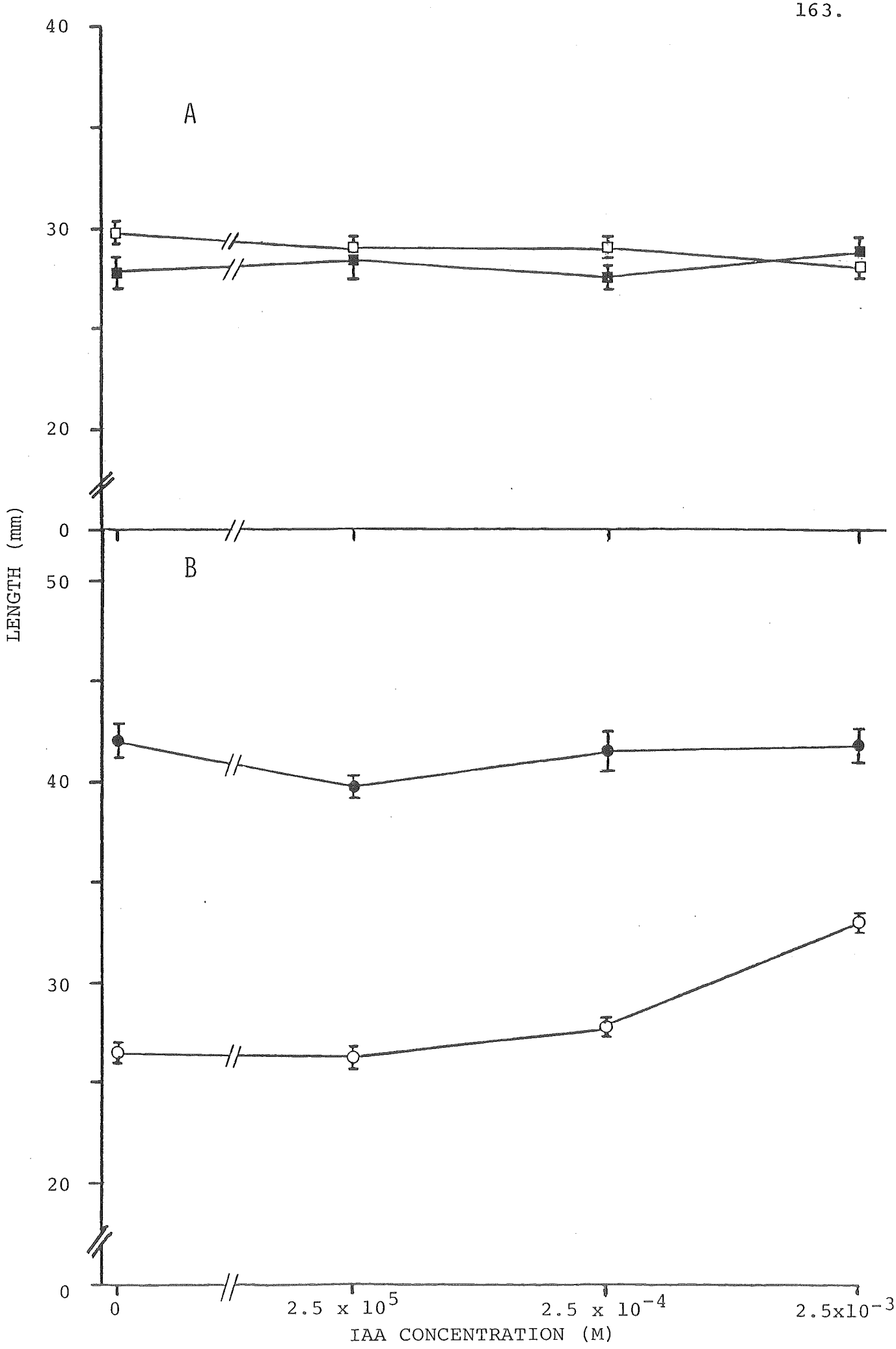
The results for both dark-grown and light-treated plants are shown in Fig. 3.56. In the control plants (i.e. injected with solvent only), the coleoptile in darkness increased in length by almost 260%, from 10.8 (above) to 27.7 mm in the 24 h period following injection (Fig. 3.56a). Exposure to red light did not affect the amount of coleoptile extension in this period, although the results in Fig. 3.56a might

Figure 3.56: Effect of injection of IAA into the endosperm, on elongation of (A) coleoptiles and (B) mesocotyls

Each value represents the mean ± standard error of 20 plants.

● ■ Dark-grown

○ □ Light-treated


A
B
LENGTH (mm)
40
30
20
0
50
40
30
20
0
0
2.5 x 10^5
2.5 x 10^-4
2.5x10^-3
IAA CONCENTRATION (M)

suggest otherwise. However the 'light' and 'dark' experiments were performed on separate occasions, and the small difference between the control coleoptile lengths in the two experiments presumably reflected slight variations in the experimental conditions in each instance. In another experiment, where etiolated and illuminated plants were grown together, lengths of 27.5 $\pm$ 0.63 mm and 27.0 $\pm$ 0.76 mm were observed for the 'dark' and 'light' coleoptiles respectively. These values are not significantly different (Student's t test, $P < 0.01$).

In the 24 h period after selection the mesocotyls of the dark-control plants increased in length by 107%, from 20.3 mm to 42.1 mm (Fig. 3.56b). In the same period, light-treated control mesocotyls elongated by 30.5% - less than 29% of the amount of extension that occurred in the dark-grown plants.

IAA injected into the endosperm of dark-grown seedlings had no significant effect on the extension of either the coleoptile or mesocotyl (Fig. 3.56, Anova, $P < 0.05$). Similarly coleoptile growth of illuminated plants was unaffected. Only extension of the photoinhibited mesocotyl was promoted by IAA, and then only at the highest test concentration (LSD, $P < 0.01$). The 6.5 mm of extra mesocotyl extension induced by 2.5×10^{-3} M IAA, represented a promotion of 105% relative to the elongation of the control plants in the 24 h following light treatment. Thus, IAA partially reversed (by about half) the inhibition resulting from exposure to light.

The experiments were each repeated twice with similar results.

The injection procedure itself did not produce a growth response in the seedlings (Table 3.22). The elongation of plants which were selected for uniformity of shoot length but not injected, did not differ significantly (LSD, $P < 0.05$) from that of plants injected with the solvent control (i.e. 0.5% aqueous ethanol). This was the case for both dark-grown seedlings and those which were exposed to red light (Table 3.22).

Table 3.22: Effect of injection of solvent control on seedling elongation

Plants were selected for length 63 h after planting and mean coleoptile, mesocotyl and root lengths determined 24 h later ('Light' and 'Dark' were separate experiments).

Treatment		Mean length (mm)		
		coleoptile	mesocotyl	root
Dark-grown	Control - no injection	27.9 ± 0.62	41.7 ± 0.91	67.5 ± 0.99
	Injected with 0.5% ethanol	27.7 ± 0.70	42.0 ± 0.82	69.2 ± 1.47
Light-treated	Control - no injection	28.9 ± 0.43	26.9 ± 0.49	67.4 ± 1.45
	Injected with 0.5% ethanol	29.8 ± 0.53	26.5 ± 0.57	69.9 ± 0.89

3.5.1.2 Application to the coleoptile tip

To compare the relative effectiveness of tip and grain application of IAA, studies were carried out in which agar impregnated with IAA was applied to the coleoptile tip of seedlings.

Plants treated in this manner exhibited pronounced downward drooping of the shoot. This response (which was observed in both dark-grown and light-treated seedlings) was not merely a result of the weight of the agar block, because plants supporting plain agar grew normally. The degree of bending was proportional to the concentration of IAA applied, which appeared to reverse the normal geotropic response of the shoot. At lower IAA concentrations, the effect was only partial, since the apical portion of the coleoptile continued to grow upwards. However, with 2.5×10^{-3} M IAA, the reversal was complete; the mesocotyl underwent a complete turn through 180° near the base and the shoot grew directly downward.

Extension growth of the shoot was promoted by IAA applied to the coleoptile tip, in both etiolated seedlings and those exposed to red light (Fig. 3.57). Coleoptile elongation, which was unaffected by IAA injected into the endosperm at the concentrations tested, was dramatically increased when IAA was applied to the coleoptile tip (Fig. 3.57a). Light treatment did not noticeably affect the response of the coleoptile, and an approximately linear relationship between coleoptile length and the logarithm of IAA concentration was evident in both light-treated and dark-grown plants. In control seedlings, coleoptile length increased by 16.2 mm during the 24 h experimental period. With 2.5×10^{-3} M IAA treatment the increase was 28.2 mm and 31.0 mm for light-treated and dark-grown coleoptiles respectively, representing growth promotions of 74% and 91% relative to the control growth increment.

Mesocotyl elongation in total darkness was promoted by IAA (Fig. 3.57b). All three test concentrations gave similar responses and all were significantly different from the control at the 5% probability level (LSD). IAA applied to the coleoptile tip, also promoted the extension of light-inhibited mesocotyls (Fig. 3.57b), with the size of the response increasing with increasing IAA concentration.

The control shown in Fig. 3.57 was a plain agar block lacking IAA, applied to the coleoptile tip. However, as an additional control (not shown in Fig. 3.57), groups of seedlings were also incubated without agar. Table 3.23 shows that the agar block itself did not significantly affect seedling extension growth (LSD, $P < 0.05$).

The above IAA application experiments were repeated on two further occasions with similar results.

Figure 3.57: Effect of application of IAA to the coleoptile tip, on elongation of (A) coleoptiles and (B) mesocotyls

(n = 20)

● ■ Dark-grown

○ □ Light-treated

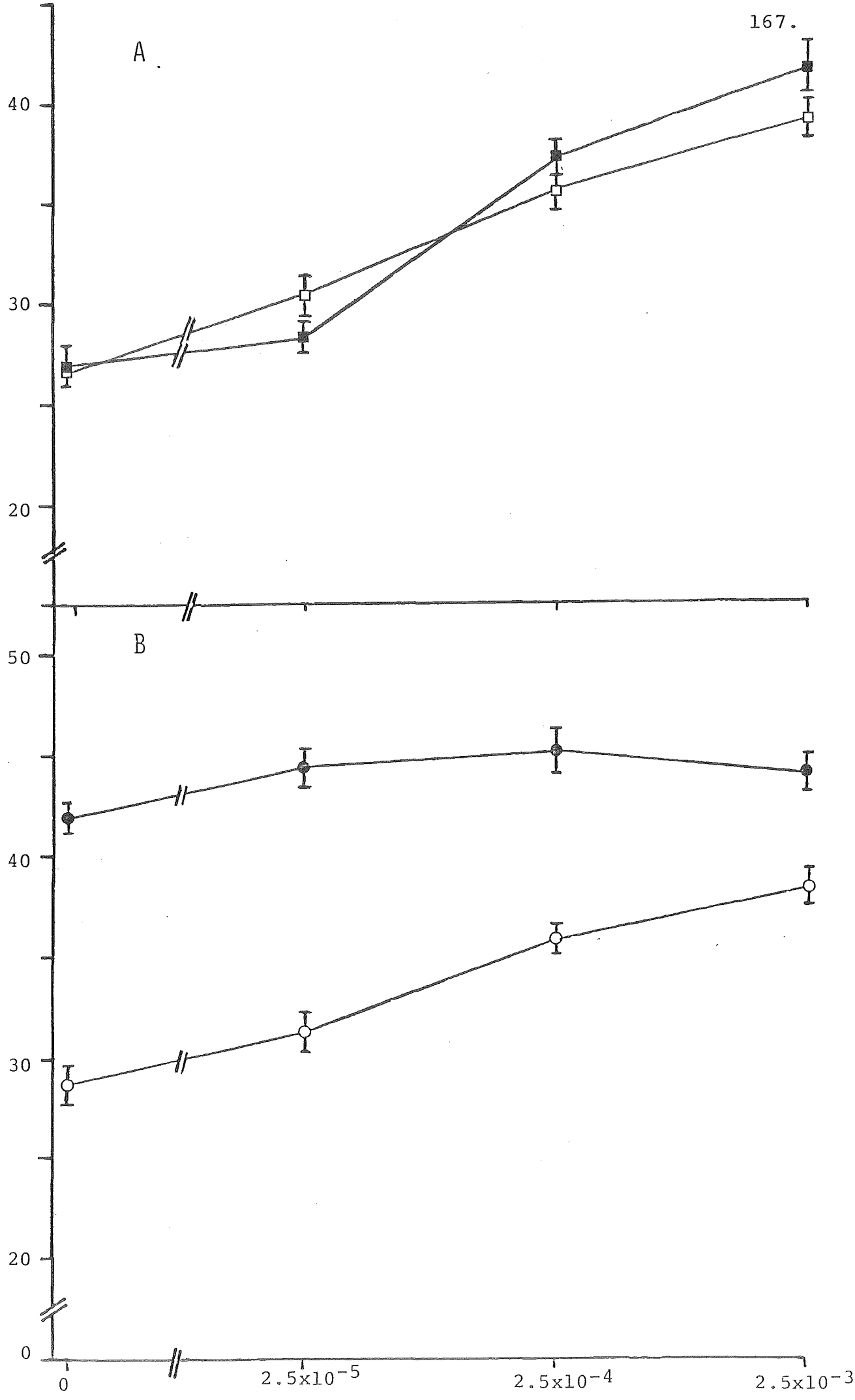

A
B
40
30
20
50
40
30
20
0
0
2.5x10^{-5}
2.5x10^{-4}
2.5x10^{-3}
IAA CONCENTRATION (M)

Table 3.23: Effect of plain agar blocks applied to the coleoptile tip, on seedling elongation

Treatment		Mean length (mm)		
		coleoptile	mesocotyl	root
Dark-grown	Control - no agar	27.6 ± 0.76	40.7 ± 0.74	66.8 ± 1.23
	Plain agar	27.0 ± 1.09	41.8 ± 0.71	68.7 ± 1.61
Light-treated	Control - no agar	27.3 ± 0.68	28.4 ± 0.75	68.4 ± 1.44
	Plain agar	26.7 ± 0.72	28.6 ± 0.87	68.9 ± 1.19

3.5.2 Other Growth Regulators

The response of Avena seedlings to applications of other growth regulators was also determined, using the endosperm-injection technique.

3.5.2.1 Gibberellic Acid

The growth response of dark-grown and light-treated Avena seedlings following injection of GA_3 is shown in Fig. 3.58. GA_3 had no significant effect on coleoptile elongation in darkness, but a slight promotion of coleoptile extension was evident in seedlings which had been exposed to light (Fig. 3.58a, Anova, $P < 0.05$).

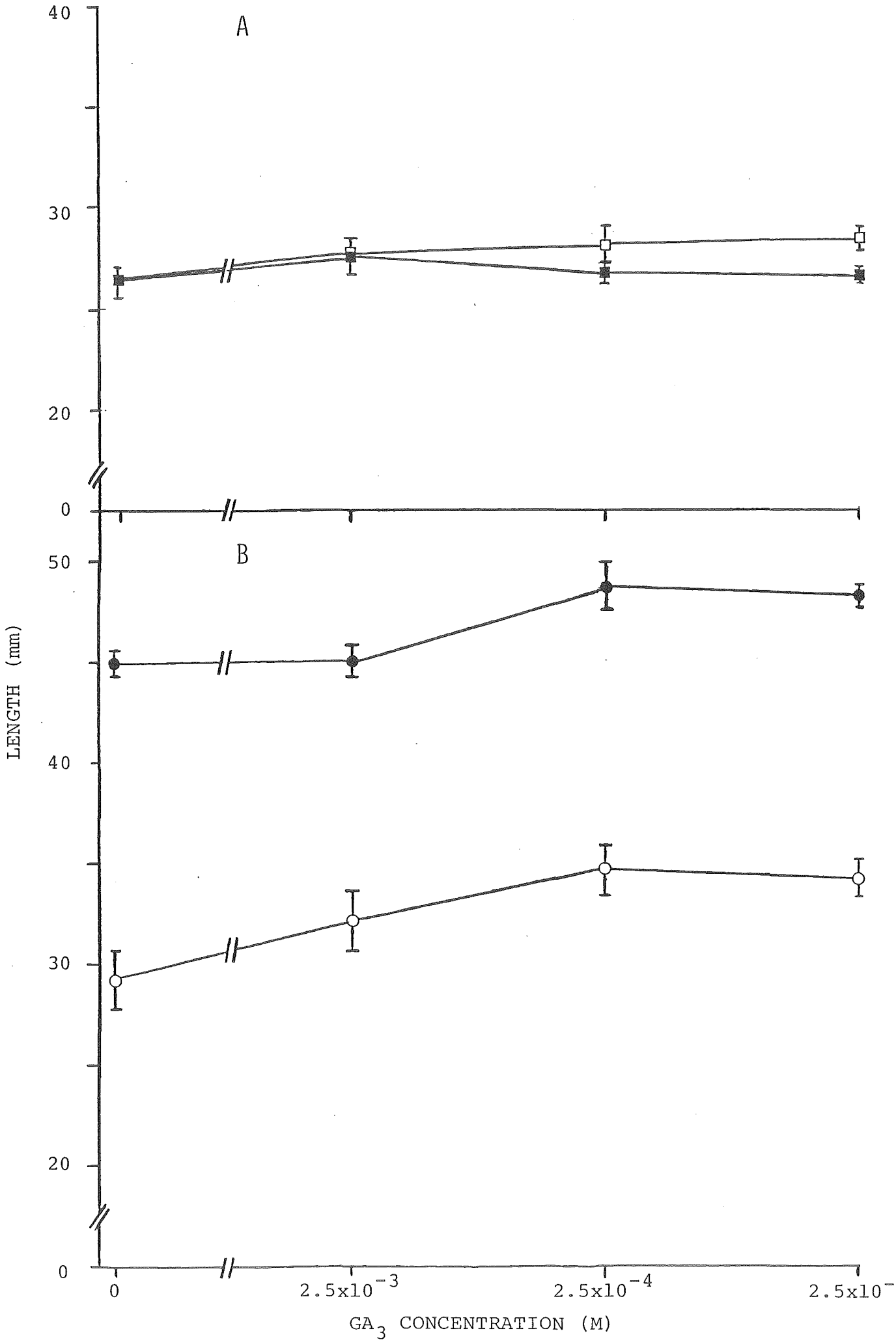
Mesocotyl extension was promoted by GA_3, irrespective of whether the plants were illuminated (Anova, $P < 0.05$). However, the response was quite variable and this is reflected in the statistical significance of the differences between the treatment means. In both cases, only the two higher test concentrations of GA_3 had a significant promotive effect on mesocotyl elongation, with 2.5×10^{-4} M GA_3 being the apparent optimum concentration (LSD, $P < 0.05$). In darkness this resulted in a 16% promotion of extension

Figure 3.58: Effect of injection of GA_3 into the endosperm, on elongation of (A) coleoptiles and (B) mesocotyls

(n = 20)

● ■ Dark-grown

○ □ Light-treated


A
B
40
30
20
0
50
40
30
20
0
LENGTH (mm)
0
2.5x10^{-3}
2.5x10^{-4}
2.5x10^{-5}
GA$_3$ CONCENTRATION (M)

relative to the control in the 24 h experimental period, while in mesocotyls which had been exposed to light the promotion of elongation was 60%.

3.5.2.2 Kinetin

Kinetin, when injected into the endosperm of either dark-grown or light-treated plants, had no significant effect on the elongation of the coleoptile or mesocotyl during the period studied (Fig. 3.59).

3.5.2.3 Abscisic Acid

Elongation of both the coleoptile and mesocotyl was markedly inhibited by ABA injected into the endosperm, whether or not the seedlings had been irradiated (Fig. 3.60). In each case an approximately linear relationship between inhibition of extension growth and ABA was evident over the range of concentrations tested. In both experiments, ABA at the highest concentration resulted in almost total inhibition of elongation, so that little or no additional extension of the coleoptile or mesocotyl occurred after the seedlings were injected (cf coleoptile and mesocotyl lengths cited at beginning of section 3.4). This effect was particularly noticeable in dark-grown mesocotyls, where the growth increment following ABA application was reduced from 23 mm in control plants, to less than 1.5 mm in plants injected with ABA at 2.5×10^{-3} M.

3.5.3 Histological Studies

Histological studies were carried out to determine the involvement of cell division and extension, in the elongation of the coleoptile and mesocotyl; and to determine how these processes were affected by treatments such as red light exposure and the application of plant growth regulators.

Only the effects of those growth regulators that promoted mesocotyl or coleoptile extension (section 3.5.1 and 3.5.2) were studied further. Three separate experiments were performed to provide seedlings for histological analysis.

Figure 3.59: Effect of injection of kinetin into the endosperm, on elongation of (A) coleoptiles and (B) mesocotyls

(n = 20)

● ■ Dark-grown

○ □ Light-treated

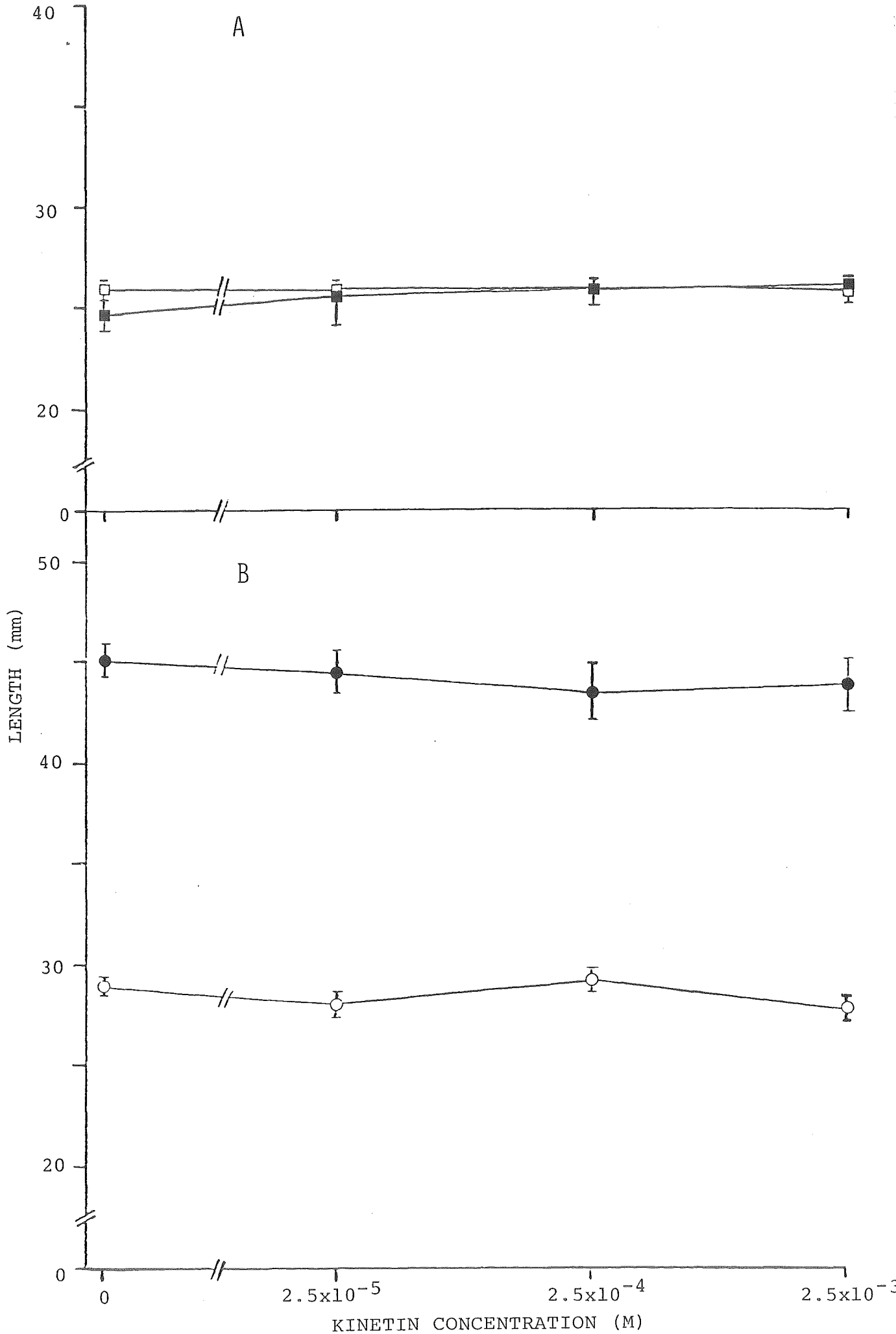
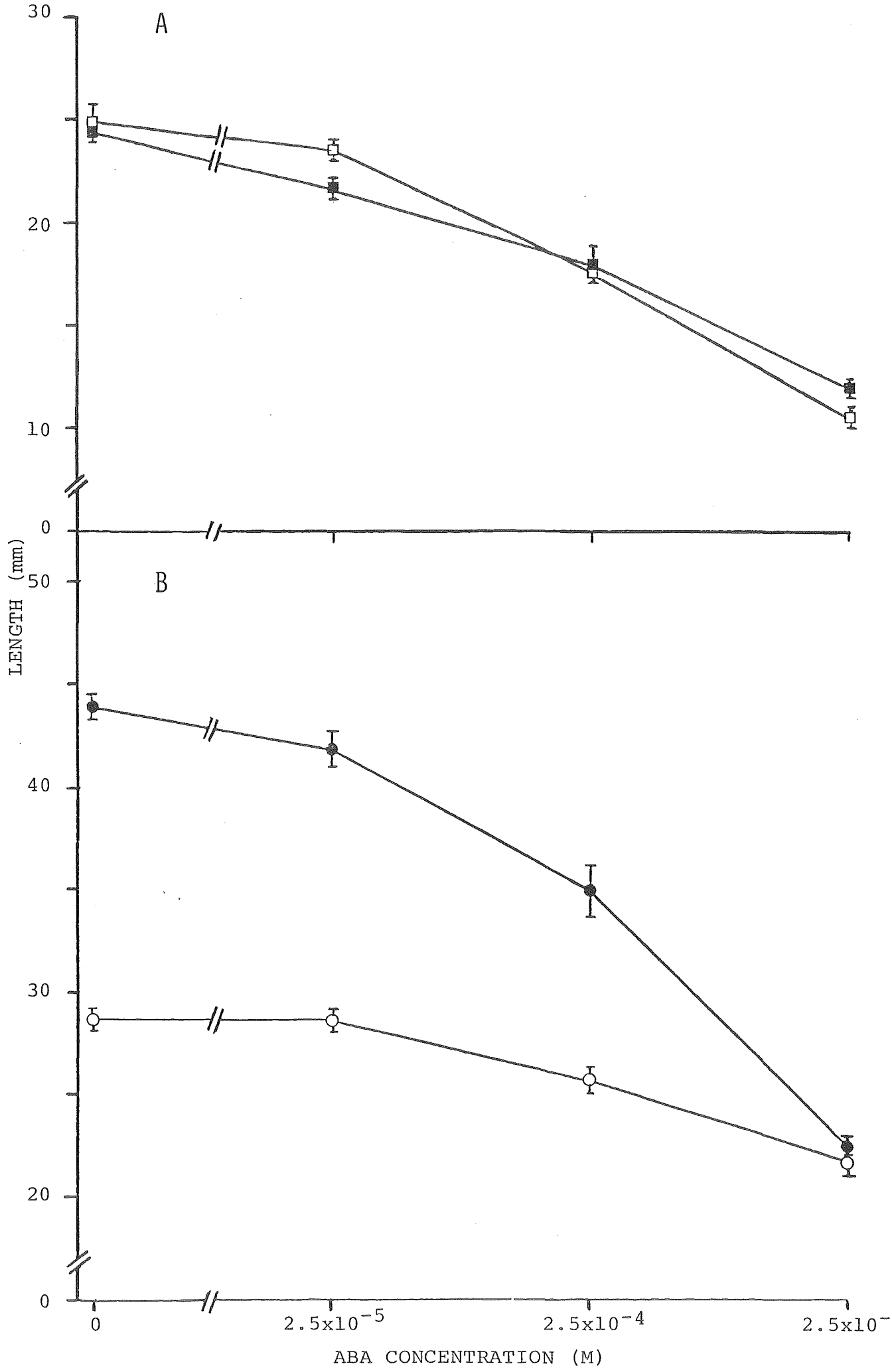

A
B
40
30
20
0
50
40
30
20
0
LENGTH (mm)
0
2.5x10-5
2.5x10-4
2.5x10-3
KINETIN CONCENTRATION (M)

Figure 3.60: Effect of injection of ABA into the endosperm, on elongation of (A) coleoptiles and (B) mesocotyls

(n = 20)

● ■ Dark-grown

○ □ Light-treated


A
B
30
20
10
0
50
40
30
20
0
LENGTH (mm)
0
2.5x10^{-5}
2.5x10^{-4}
2.5x10^{-}
ABA CONCENTRATION (M)

In one, plants were grown for cell studies on: (a) 63 h old seedlings selected for uniformity of length (b) 87 h old dark-grown seedlings, with or without IAA applied to the coleoptile tip, and (c) 87 h old light-treated seedlings with or without IAA applied to the coleoptile tip. In a second experiment light-treated control plants, and those injected with IAA and GA_3 were grown in the usual manner (section 3.5) and harvested when aged 87 h. The final experiment involved growing seedlings to examine the effect of GA_3 injection on coleoptile and mesocotyl extension in darkness. Because some of the growth regulator treatments used a common control, the control data in some cases are represented in two different graphs and tables.

The growth regulators were applied at concentrations of 2.5×10^{-3} M in all cases. Representative seedlings were sampled, embedded and sectioned as described in section 2.6.3. The mean number of cells per vertical column in the coleoptile and in the mesocotyl was determined, as well as mean cell length per millimetre along the length of each structure (section 2.6.3).

3.5.3.1 Coleoptile development

The coleoptile, 63 h after imbibition, contained columns of, on average, 157 cells (Table 3.24), increasing progressively in length from 0.036 mm at the coleoptile tip to approximately 0.1 mm near the coleoptilar node (Fig. 3.61). Between 63 h and 87 h after planting, the coleoptile grown in darkness, increased in length by about 260% (from 10 mm to 26 mm). This was mainly the result of the extension of cells which already existed in the coleoptile 63 h after imbibition, since cell numbers increased by only 9% to 172 cells (Table 3.24).

Two groups of cell sizes were evident in the coleoptile after 87 h (Fig. 3.61). Those in the apical half showed a linear increase in length, from 0.05 mm to 0.24 mm, with increasing distance from the tip; whereas cell length was approximately constant, at 0.24 mm, in the basal half. Nearly

Figure 3.61: Distribution of cell length along the coleoptiles of 63 h old dark-grown plants, and of 87 h old plants as modified by illumination

● 63 h old, dark-grown
■ 87 h old, dark grown
□ 87 h old, briefly illuminated when aged 63 h

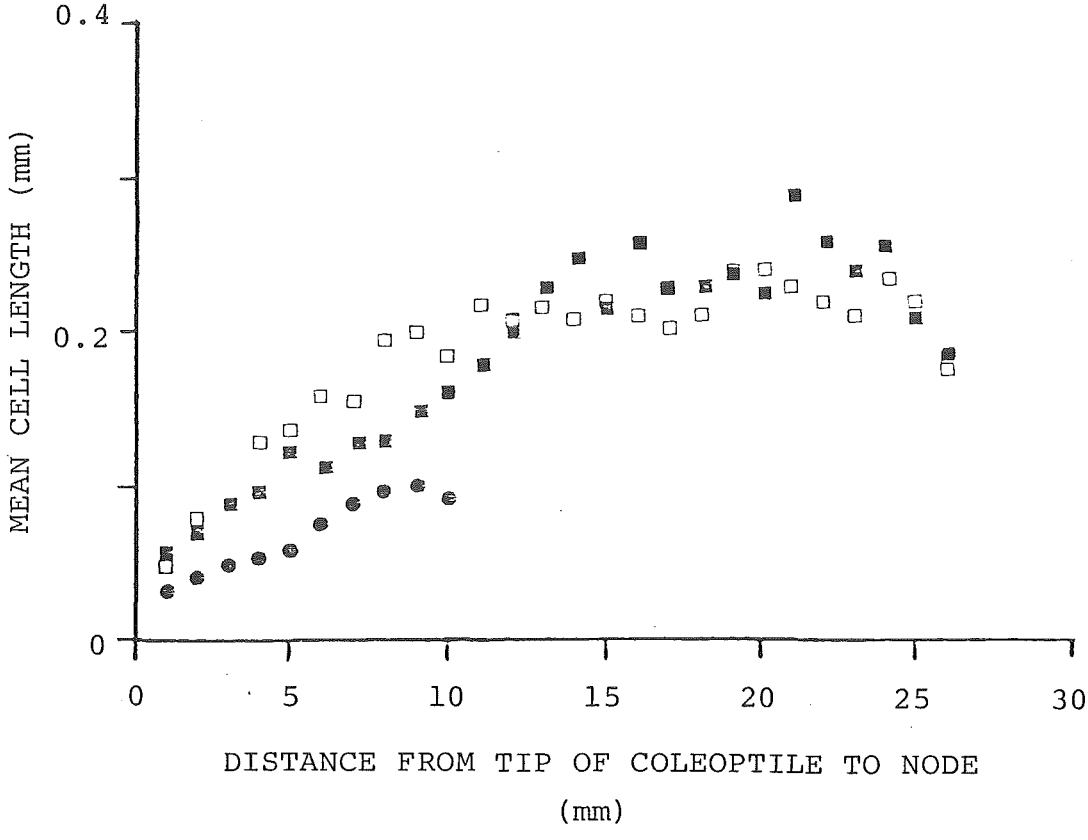

0.4
0.2
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
DISTANCE FROM TIP OF COLEOPTILE TO NODE
(mm)

Table 3.24: Mean cell numbers per column in coleoptiles and mesocotyls of 63 h old dark-grown plants, and of 87 h old plants as modified by illumination

Seedling Treatment	Mean cell number per column	
	coleoptile	mesocotyl
63 h old, dark-grown	157 ± 5	93 ± 4
87 h old, dark-grown	172 ± 7	126 ± 4
87 h old, briefly illuminated when aged 63 h	162 ± 10	109 ± 5

30% of the 172 cells in the coleoptile at this stage, were contained in the lower half.

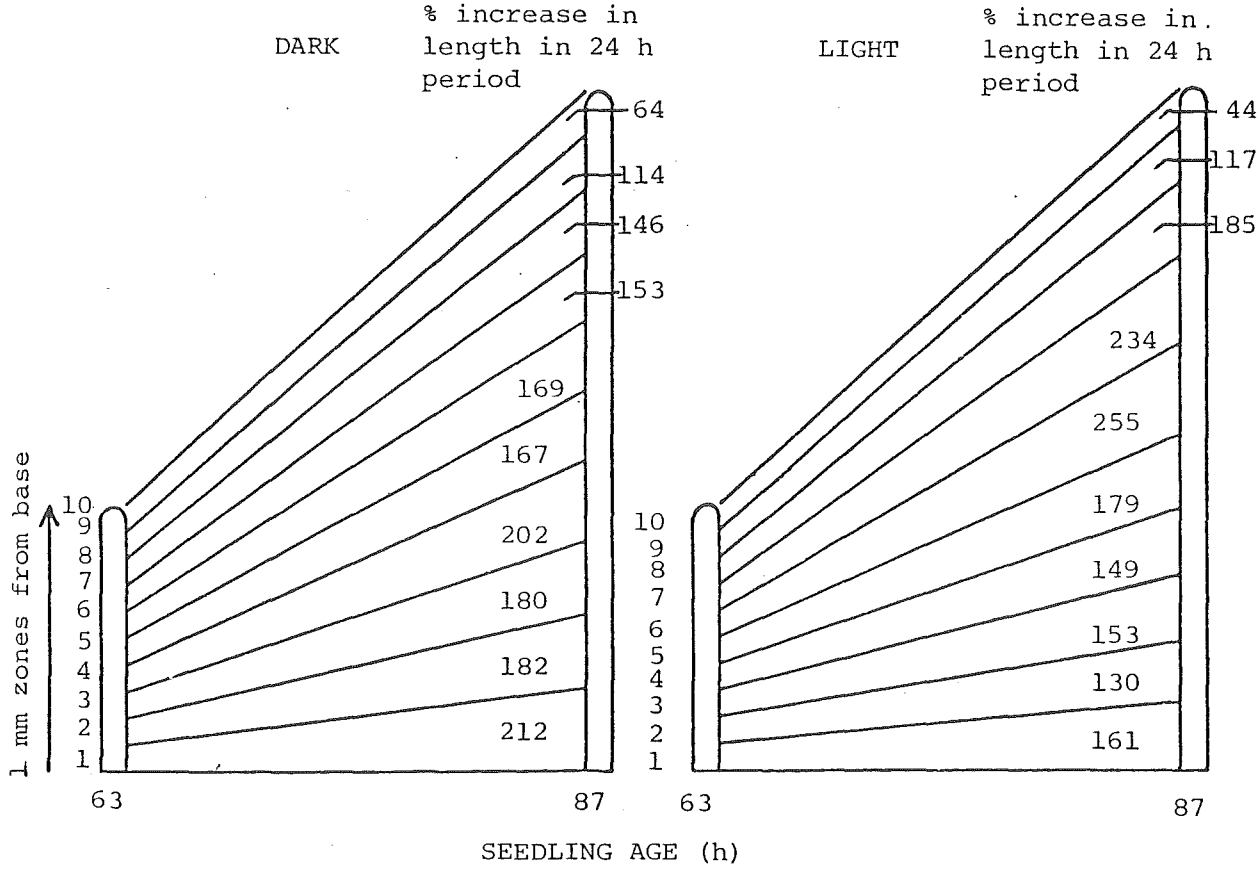
Fig. 3.62a shows the amount of elongation undergone by each millimetre of tissue in the 63 h old coleoptile, when grown in darkness. From the study undertaken, it was not possible to determine where in the coleoptile the new cells were being formed - only that total cell numbers were changing. However, according to Avery and Burkholder (1936) and Mer (1969) sporadic mitoses occur throughout the length of the coleoptile. It was assumed that a similar situation would exist in the coleoptiles used in this investigation and that a 9% increase in cell numbers would occur in each zone of the 63 h old coleoptile. Therefore, in deriving Fig. 3.62a, the mean increase in cell length was calculated for each sector of the coleoptile and this figure was then multiplied by 1.09 to reflect the contribution made by new cells.

The greatest elongation occurred in the 4 basal zones of the coleoptile, where an average of 2 mm of new tissue was produced by each one millimetre zone of the 63 h old coleoptile (Fig. 3.62a). Towards the coleoptile tip extension declined gradually and, in zones 1 and 2, growth increaments of 0.64 mm and 1.14 mm respectively, were observed.

Figure 3.62: Elongation of 1 mm zones of the 63 h old dark-grown coleoptile in the subsequent 24 h period

The figures to the left of the 87 h old seedling indicate the percentage increase in length that occurred in the 24 h period

A. Grown in darkness
B. Briefly illuminated when aged 63 h


DARK
% increase in length in 24 h period
64
114
146
153
169
167
202
180
182
212
LIGHT
% increase in length in 24 h period
44
117
185
234
255
179
149
153
130
161
1 mm zones from base
10
9
8
7
6
5
4
3
2
1
63
87
SEEDLING AGE (h)

Although coleoptiles which had been exposed to red light showed similar total increases in length in the subsequent 24 h period, to dark-grown coleoptiles, differences were apparent at the cellular level (Fig. 3.61 above). Cells in the upper half of light-treated coleoptiles were longer than in etiolated plants and the gradient of increase in cell length away from the coleoptile tip was steeper. Consequently, 10 mm behind the coleoptile tip, cells in the illuminated coleoptiles exceeded 0.20 mm in length, whereas at the same point in dark-grown seedlings, cells were 0.16 mm long.

In the basal half of the coleoptile, light-treated cells tended to be shorter, averaging 0.21 mm, rather than 0.24 mm as was the case in darkness. These changes in cell length counterbalanced one another and meant that overall, no difference in the length of dark-grown and light-treated coleoptiles was apparent 87 h after planting. Cell division, in the period studied, was not significantly affected by light-treatment (Student's t-test, $P < 0.05$) (Table 3.24).

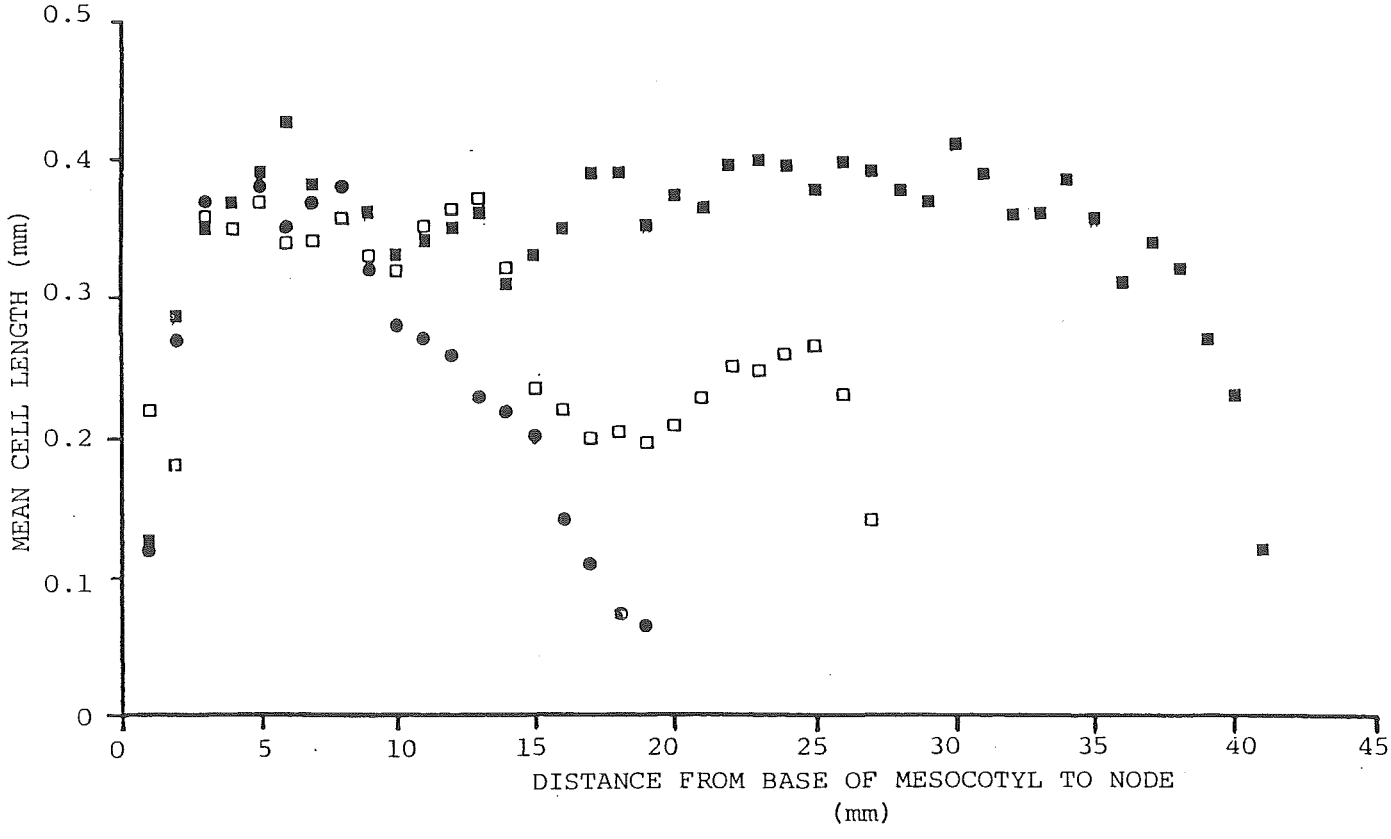
Comparison of Figs. 3.62a and b shows how the extension of the various parts of the coleoptile was affected by light exposure. Extension in the upper 60% of the coleoptile was promoted by light, except near the coleoptile tip, where a slight reduction was observed. The cells most responsive to promotion by light were located in the 4th and 5th zones behind the coleoptile tip. Between them, these two zones contributed 4.9 mm of new tissue in the 24 h following illumination, compared with 3.2 mm for the same sectors in dark-grown plants. In the basal 4 mm of the coleoptile, light inhibited the extension of cells, and the average growth increment of these zones was 25% less than in plants grown in darkness.

3.5.3.2 Mesocotyl development

The cortex of 63 h old dark-grown mesocotyls, on average, comprised columns of 93 cells (Table 3.24 above). The basal 8 mm of the mesocotyl, which constituted 42% of the total length, contained only 28% of the cells, reflecting the greater length of cells in this region (Fig. 3.63). Most

Figure 3.63: Distribution of cell length along the mesocotyls of 63 h old dark-grown plants, and of 87 h old plants as modified by illumination

● 63 h old, dark-grown
■ 87 h old, dark-grown
□ 87 h old, briefly illuminated when aged 63 h


0.5
0.4
0.3
0.2
0.1
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
40
45
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

of the cells in the basal region were about 0.35 - 0.38 mm long, but near the scutellum, cell length declined abruptly. From about 8 mm from the base of the mesocotyl, towards the coleoptilar node, cells became progressively shorter, decreasing from 0.35 mm to 0.06 mm in length.

By 87 h after planting, mesocotyls grown in darkness had achieved a mean length of 41 mm and, with the exception of the region within 6 mm of the coleoptilar node, comprised cells with lengths of between 0.33 and 0.40 mm (Fig. 3.63). Near the node, cell length declined sharply to 0.12 mm. The 22 mm increase in mesocotyl length that occurred between the 63 h and 87 h harvest times was achieved, partly by a 35% increase in cell numbers, from 93 to 126 (Table 3.24), and partly by extension of existing cells in the 63 h old structure (Figs. 3.63 and 3.64).

In constructing Fig. 3.64 a and b it was assumed that new cell formation was confined to the 3 mm zone immediately below the coleoptilar node. This was based on the observations of Causton and Mer (1966) that mesocotyl cells lost the capacity to divide when a length of 0.12 mm was reached. All cells, except those in the upper 3 mm of the mesocotyl, had exceeded this length by 63 h after imbibition.

No change in the length of the cells in the basal 8 mm of the mesocotyl occurred over the 24 h study period (Fig. 3.64a). However, apical to this, the expansion of each 1 mm zone was greater, closer to the coleoptilar node. Growth increments in the 24 h period, increased from 13% in the 9th sector from the base, to 770% in the zone just below the coleoptilar node. Since all cells achieved an approximately similar length by the end of the experiment, the expansion of each zone was a reflection of the size of the cells within that zone when the plants were selected, and hence the extra growth necessary for the cells to reach their mature length. In the three zones immediately below the coleoptilar node, cell division also contributed towards new growth, but even so, elongation of existing cells accounted for 70% of the extension in these sectors. Slightly more than half the 87 h old dark-grown mesocotyl was derived from this upper 3 mm of tissue in the 63 h old mesocotyl.

Mesocotyls exposed to red light, elongated slightly less than 7 mm in the 24 h period that followed, to reach a length of 27 mm (Fig. 3.63). In contrast, over the same period, dark-grown mesocotyls (Fig. 3.63) increased in length by 22 mm to 41 mm. This light inhibition of mesocotyl elongation was the result of a combination of reduced cell division (Table 3.24 above), and the limited extension of certain cells in the mesocotyl (Fig. 3.63).

In the 24 h period following illumination, cell numbers increased by 17%, which was slightly less than half the rate of cell production that occurred in dark-grown mesocotyls (Tabel 3.24). Cells in the basal half of the light-treated mesocotyl 87 h after planting, were of similar length to those in plants grown in darkness (Fig. 3.63). However, in the upper half of the mesocotyl, cells exposed to light were much shorter than their dark-grown counterparts. A cluster of particular short cells, about 0.20 mm long, was situated 16 to 21 mm from the base of the mesocotyl. Between these short cells and the coleoptilar node, cell length increased slightly to 0.26 mm, and then declined to 0.14 mm in the region of the node itself.

Light had the greatest inhibitory effect on the elongation of the 14th and 15th zones of the 63 h old mesocotyl (Fig. 3.64b cf a). Cells in these 2 zones, which were about 0.20 mm to 0.21 mm long at the time of illumination, extended only slightly in the subsequent 24 h period. Cells basal to these, which were longer than 0.21 mm at the time of exposure, were unaffected by light treatment. Consequently, the extension of the basal 13 mm of the mesocotyl was similar in both dark-grown and light-treated plants.

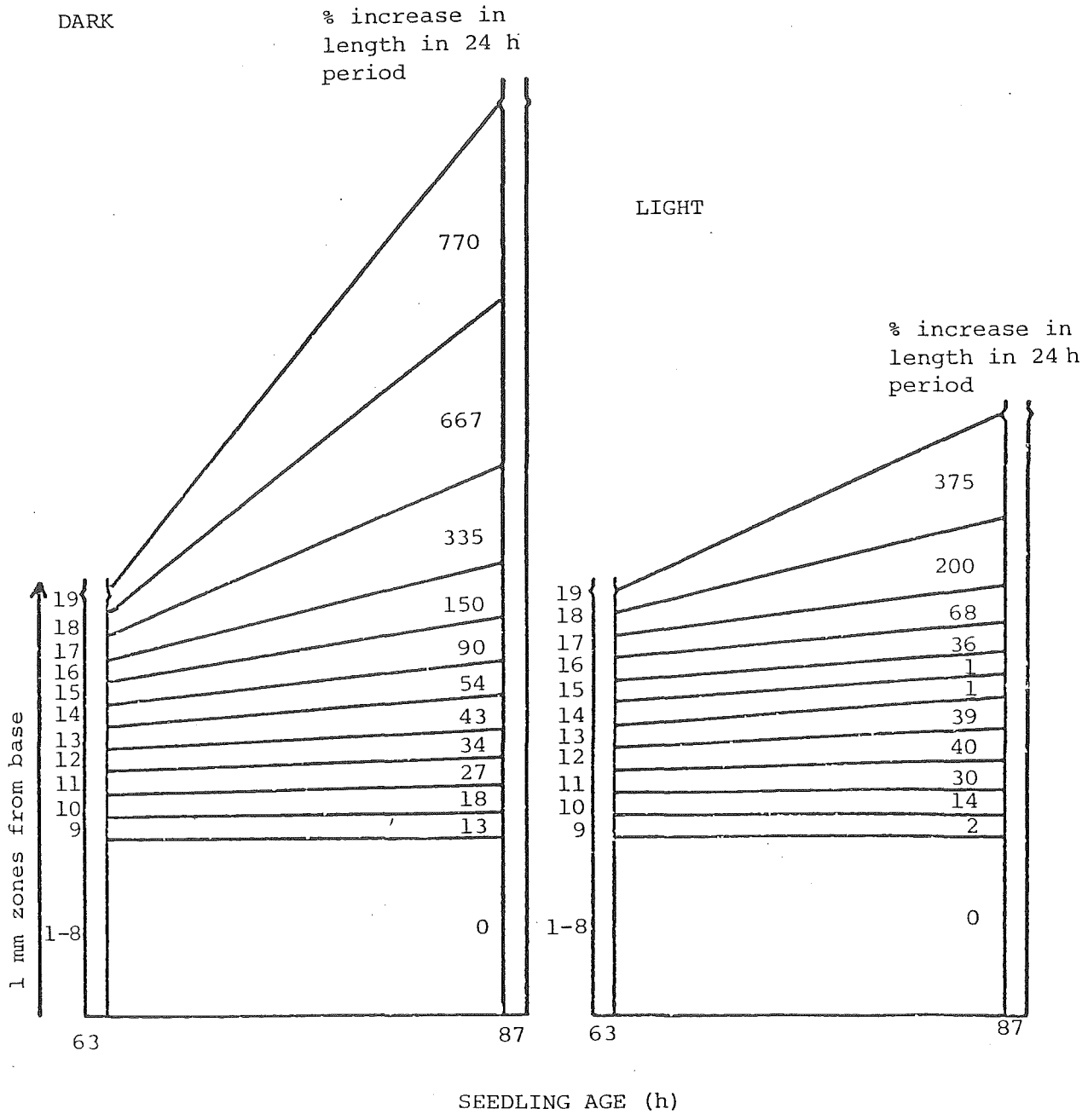
Apical to the 14th and 15th zones, partial inhibition of cell elongation as a result of light treatment was observed. The susceptability of cells to light inhibition decreased with proximity to the coleoptilar node (and diminishing cell length).

Adjacent to the coleoptilar node, where average cell length was 0.06 mm at the time of illumination, extension of the zone was inhibited by 50%. Three millimetres below

Figure 3.64: Elongation of 1 mm zones of the 63 h old dark-grown mesocotyl in the subsequent 24 h period

The figures to the left of the 87 h old seedling indicate the percentage increase in the length that occurred in the 24 h period.

A. Grown in darkness
B. Briefly illuminated when aged 63 h


DARK
% increase in length in 24 h period
770
667
335
150
90
54
43
34
27
18
13
0
19
18
17
16
15
14
13
12
11
10
9
1-8
1 mm zones from base
63
87
LIGHT
% increase in length in 24 h period
375
200
68
36
1
1
39
40
30
14
2
0
19
18
17
16
15
14
13
12
11
10
9
1-8
63
87
SEEDLING AGE (h)

the node, cells were 0.14 mm long when illuminated, and extension was inhibited by 75% (Fig. 3.64b cf. a).

3.5.3.3 Effect of IAA applied to coleoptile tip

(i) *Dark-grown plants.* IAA applied to the coleoptile tip in agar capsules promoted the extension of coleoptiles grown in darkness (Fig. 3.65, and section 3.5.1.2 above). After 87 h incubation, IAA-treated coleoptiles were 39 mm long compared to 26 mm for coleoptiles from control plants. This promotion resulted from an increase in mean cell length (Fig. 3.65), however cell numbers were not affected by IAA treatment (Table 3.25).

The extension of all cells in the coleoptile was promoted by IAA. Cells treated with IAA on average increased in length by 350% in the 24 h experimental period, whereas untreated cells increased by 245%.

The slight promotion of mesocotyl elongation in darkness, following IAA treatment (Fig. 3.66), appeared to result from an increase in cell division only. (Table 3.25). However, the change in cell numbers was not statistically significant (Student's t test).

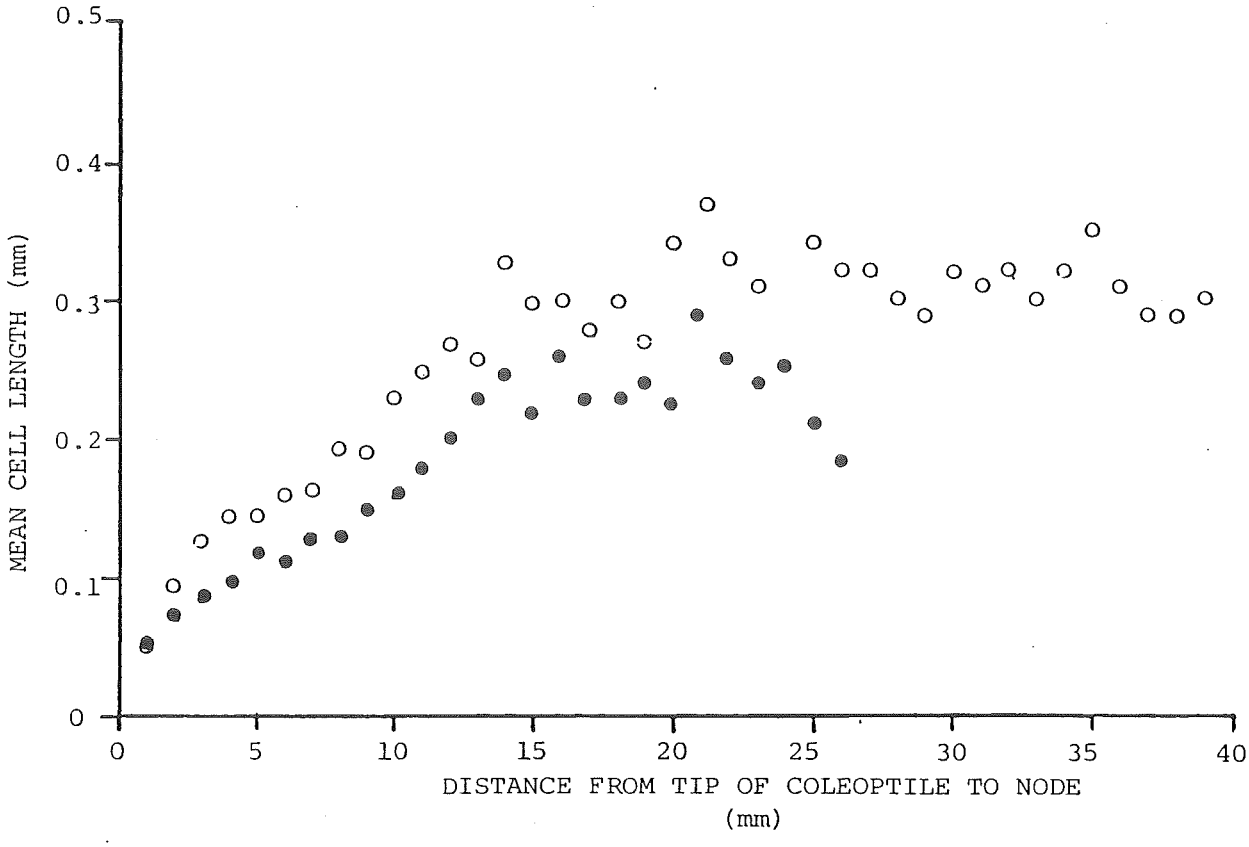
Table 3.25: Mean cell number per column in coleoptiles and mesocotyls of 87 h old plants as modified by illumination and IAA application to the coleoptile tip

Seedling Treatment	Mean cell number per column	
	Coleoptile	Mesocotyl
Dark-grown, - IAA	172 ± 7	126 ± 4
Dark-grown, + IAA	170 ± 17	135 ± 8
Illuminated, - IAA	162 ± 10	109 ± 5
Illuminated, + IAA	164 ± 4	127 ± 14

(ii) *Light-treated plants.* The extension of coleoptiles which had been exposed to red light was promoted by IAA application (Fig. 3.67 and section 3.5.1.2, above). As with

Figure 3.65: Distribution of cell length along the coleoptiles of 87 h old dark-grown plants as modified by IAA applied to the coleoptile tip.

Figure 3.66: Distribution of cell length along the mesocotyls of 87 h old dark-grown plants as modified by IAA applied to the coleoptile tip


0.5
0.4
0.3
0.2
0.1
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
40
DISTANCE FROM TIP OF COLEOPTILE TO NODE
(mm)

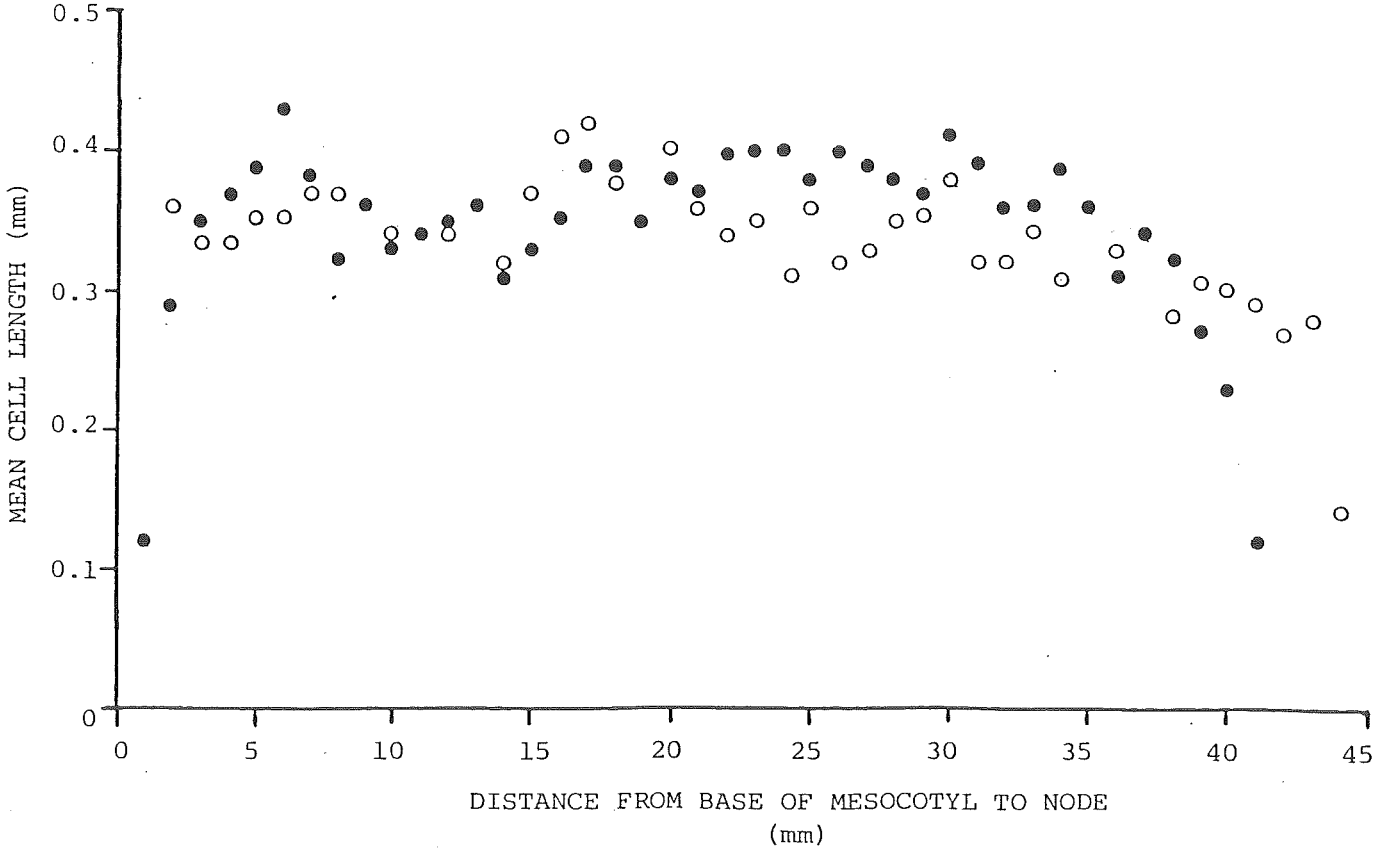
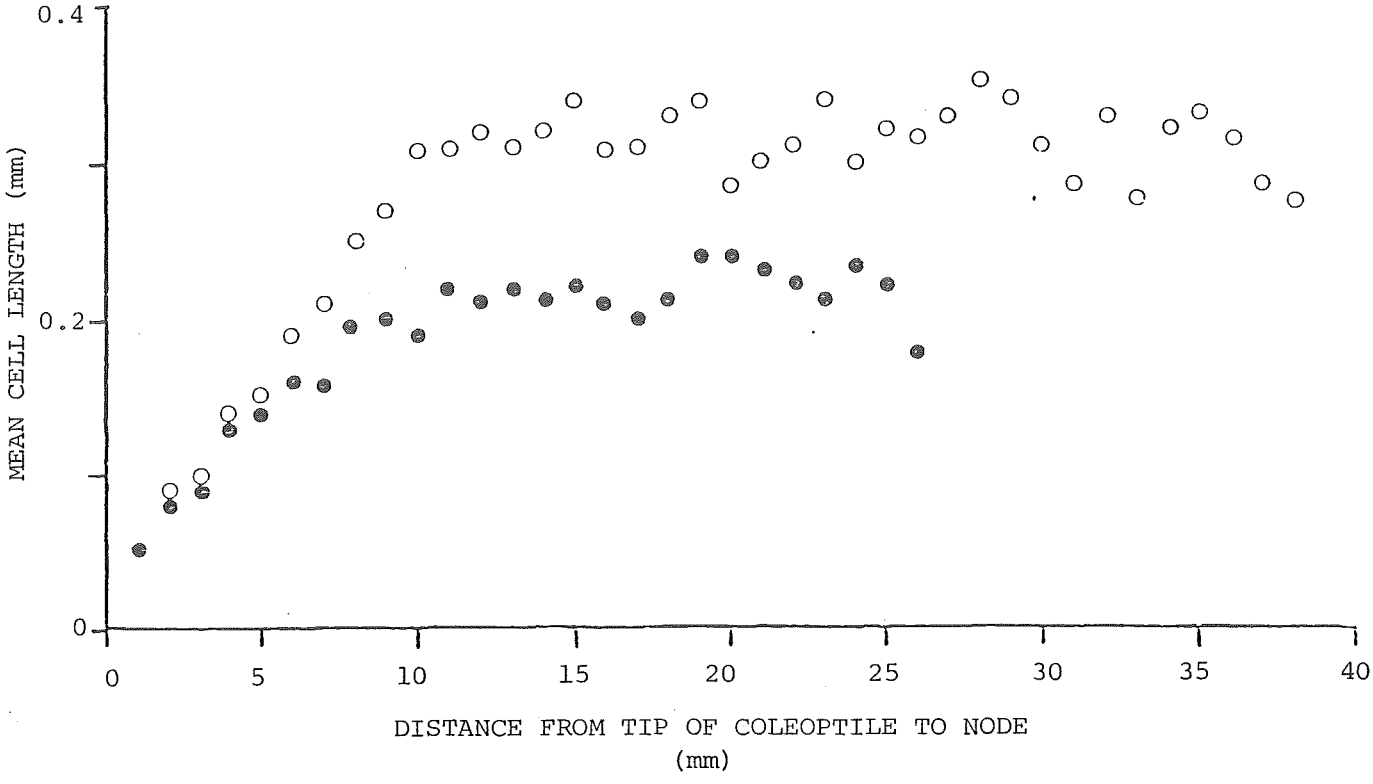

0.5
0.4
0.3
0.2
0.1
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
40
45
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

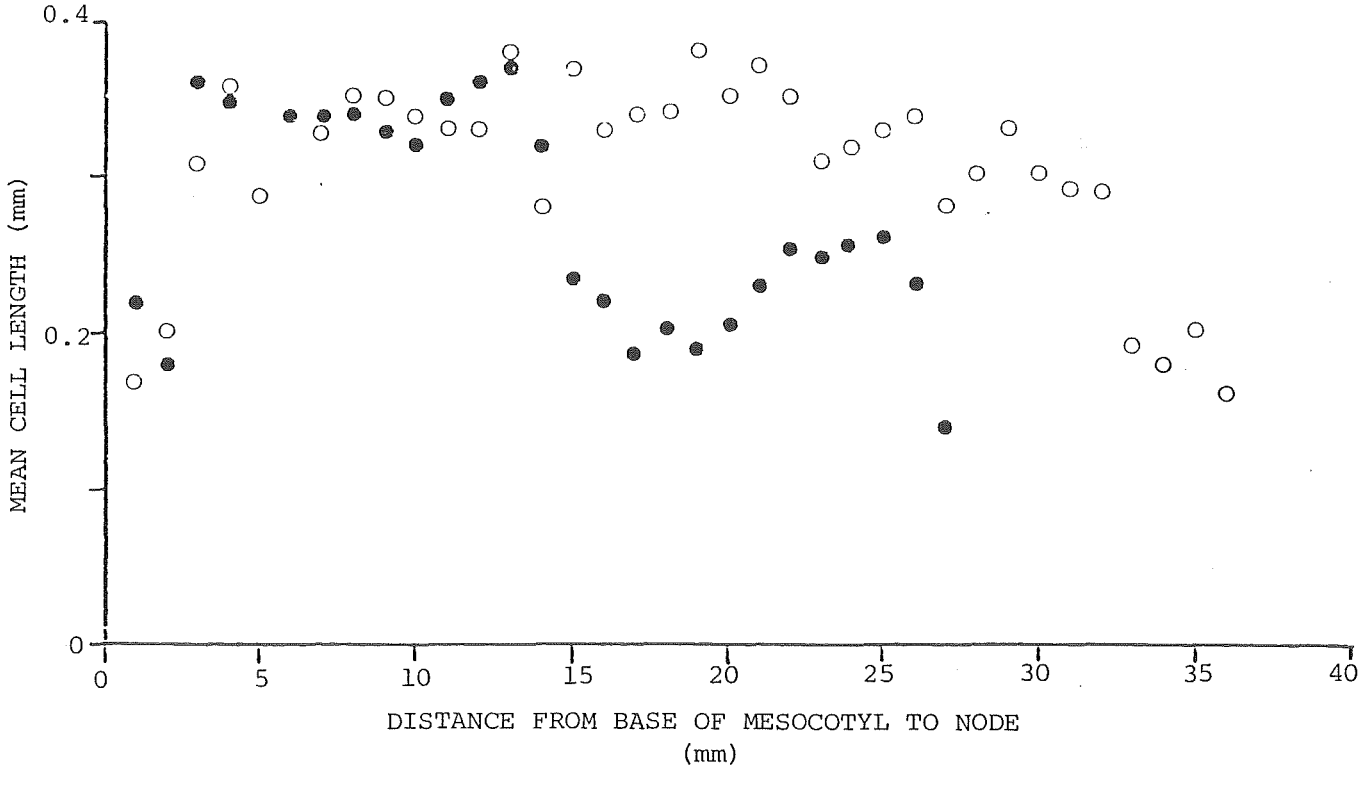
Figure 3.67: Distribution of cell length along the coleoptiles of 87 h old light-treated plants as modified by IAA applied to the coleoptile tip.

● Control
○ IAA

Figure 3.68: Distribution of cell length along the mesocotyls of 87 h old light-treated plants as modified by IAA applied to the coleoptile tip

● Control
○ IAA


0.4
0.2
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
40
DISTANCE FROM TIP OF COLEOPTILE TO NODE
(mm)


0.4
0.2
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
40
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

dark-grown coleoptiles, IAA acted by increasing cell elongation (Fig. 3.67) without affecting cell division (Table 3.25). Within 5 mm of the coleoptile tip, cell extension was unaffected by IAA. However in the rest of the coleoptile, extension was promoted; and cells averaged 0.31 mm in length, compared to 0.26 mm in light-treated coleoptiles which did not receive IAA.

IAA applied to the coleoptile tip almost fully reversed the inhibitory effect of red light on mesocotyl extension (Fig. 3.68 and section 3.5.1.2 above). Cell division was restored to a level comparable to dark-grown, untreated mesocotyls (Table 3.25), and the elongation of cells which had been inhibited by light was stimulated by IAA (Fig. 3.68). Cells, which after light treatment grew to only about 0.20 mm in length, elongated to about 0.35 mm, when IAA was applied. This was approximately the same size as cells in control mesocotyls, grown in darkness. However, a number of cells within 4 mm of the coleoptilar node did not exceed 0.20 mm in length. The shortness of these 20 or so cells accounted for the small discrepancy in length between dark-grown mesocotyls which did not receive IAA, and those which had been exposed to light and treated with exogenous IAA.

3.5.3.4 Effect of IAA applied to endosperm

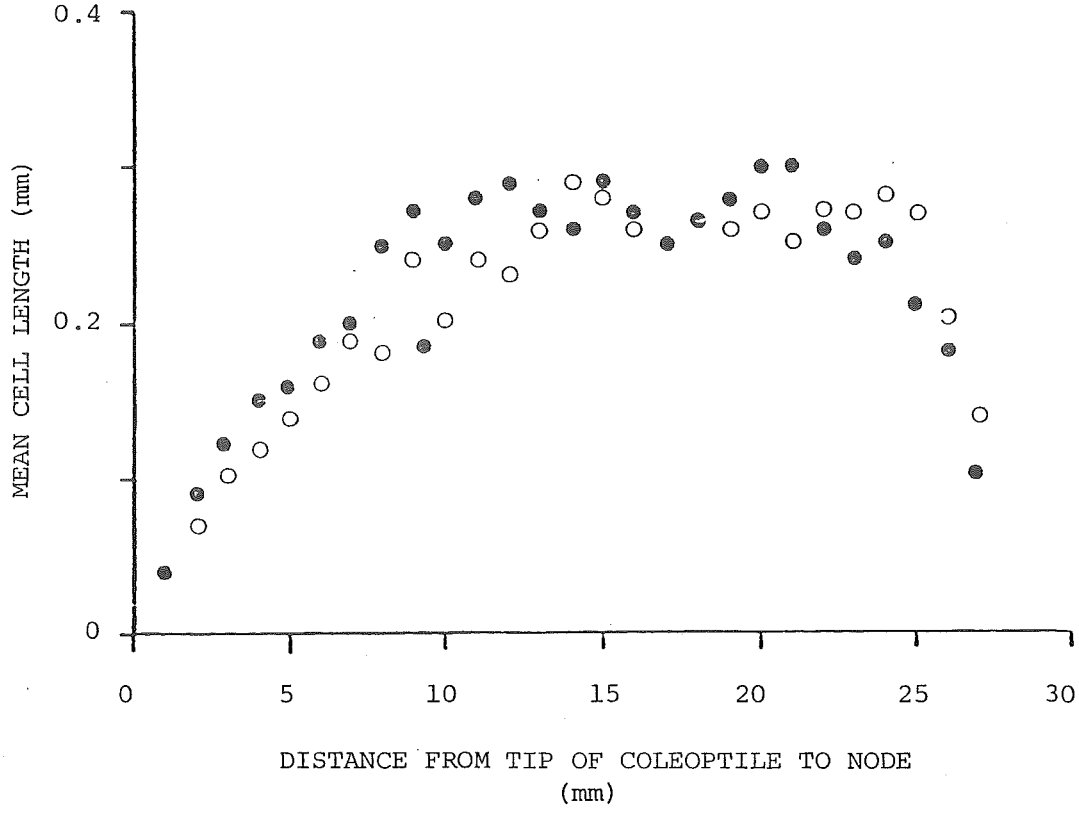
Histological studies were also carried out to determine the mechanism by which exogenous IAA promoted mesocotyl elongation, following its application to the endosperm of light-treated plants. Overall coleoptile extension was unaffected by IAA injected into the endosperm (Fig. 3.69 and section 3.5.1.1 above). Similarly, no response was evident at the cellular level. The small difference in cell numbers between IAA-treated and control coleoptiles was not statistically significant (Table 3.26, Student's t test, $P < 0.05$), and no obvious difference in cell length was observed (Fig. 3.69).

Figure 3.69: Distribution of cell length along the coleoptiles of 87 h old light-treated plants as modified by IAA injected into the endosperm

● Control
o IAA

Figure 3.70: Distribution of cell length along the mesocotyls of 87 h old light-treated plants as modified by IAA injected into the endosperm

● Control
o IAA


MEAN CELL LENGTH (mm)
0.4
0.2
0
0
5
10
15
20
25
30
DISTANCE FROM TIP OF COLEOPTILE TO NODE
(mm)

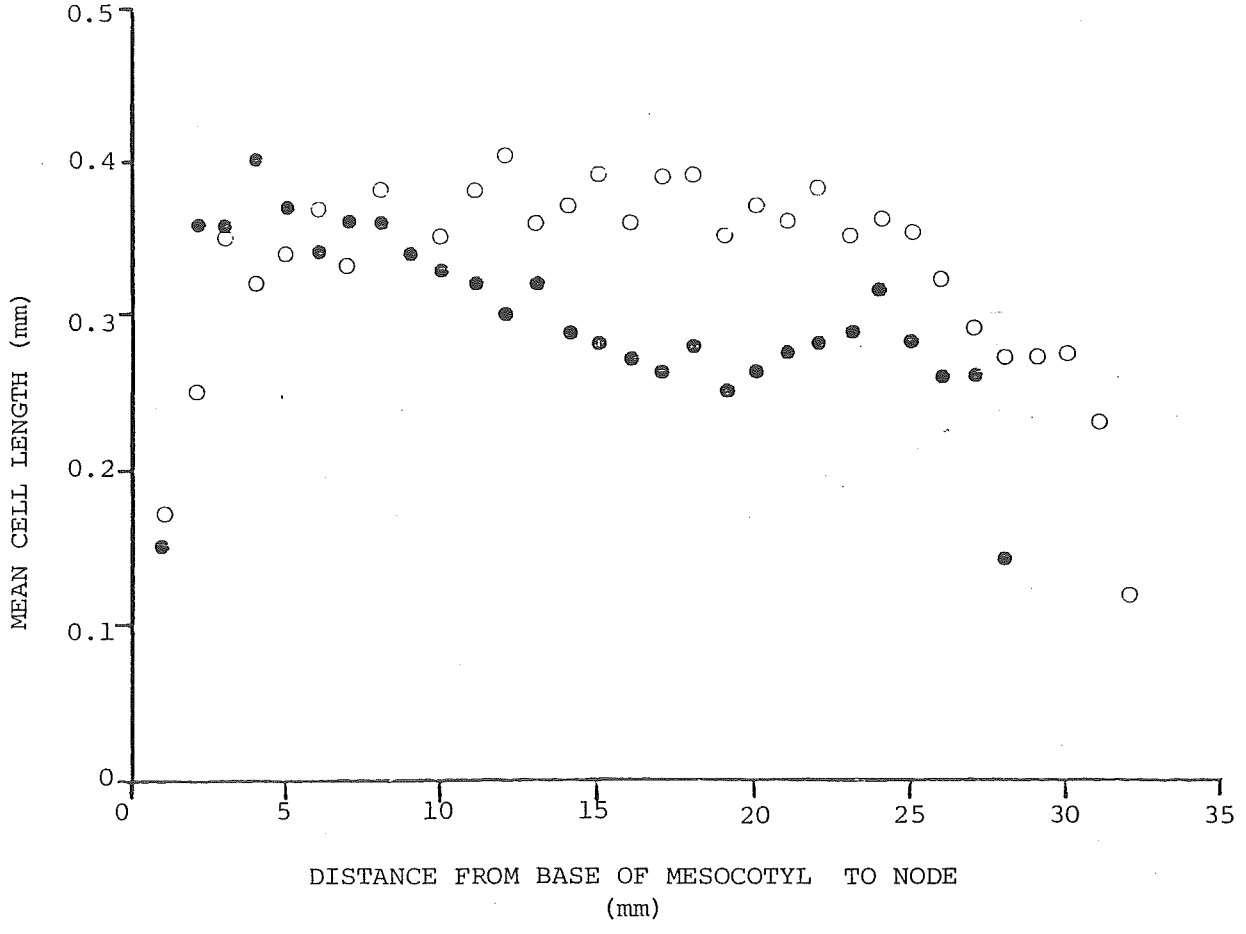

MEAN CELL LENGTH (mm)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

Fig. 3.26: Mean cell number per column in coleoptiles and mesocotyls of 87 h old light-treated plants as modified by IAA injected into the endosperm.

Seedling Treatment	Mean cell number per column	
	Coleoptile	Mesocotyl
Control (without IAA)	148 ± 11	99 ± 14
IAA	157 ± 7	104 ± 11

Light inhibition of mesocotyl extension was partially reversed by IAA injected into the endosperm (Fig. 3.70 and section 3.5.1.1, above). Cell division was unaffected by IAA treatment (Table 3.26, Student's t test $P < 0.05$); however, the elongation of the cluster of short cells, 10 to 24 mm from the base of the mesocotyl, was promoted (Fig. 3.70). Following IAA treatment, these cells achieved lengths of between 0.35 and 0.40 mm, compared with 0.25 to 0.30 mm for light-exposed cells without IAA treatment. Cell extension in the basal portion of the mesocotyl, and near the coleoptilar node was unaffected by IAA application.

3.5.3.5 Effect of GA_3 applied to endosperm

(i) *Dark-grown plants.* GA_3 injected into the endosperm of dark-grown seedlings had no effect on either cell division or cell elongation in the coleoptile (Table 3.27 and Fig. 3.71), but promoted cell extension in the mesocotyl (Fig. 3.72). The small difference in cell numbers in control and GA_3-treated mesocotyls was not statistically significant (Table 3.27, Student's t test $P < 0.05$).

(ii) *Light-treated plants.* Coleoptiles of plants which had been exposed to red light were also unaffected by GA_3 application to the endosperm (Fig. 3.73 and Table 3.28), but mesocotyl elongation was promoted by 11% (Fig. 3.74).

Figure 3.71: Distribution of cell length along the coleoptiles of 87 h old dark-grown plants as modified by GA_3 injected into the endosperm

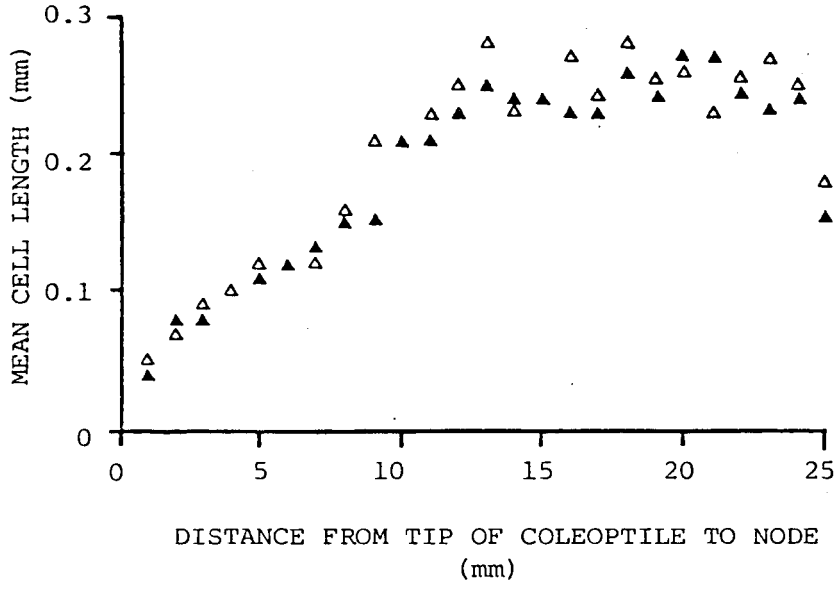
▲ Control

△ GA_3

Figure 3.72: Distribution of cell length along the mesocotyls of 87 h old dark-grown plants as modified by GA_3 injected into the endosperm

▲ Control

△ GA_3


MEAN CELL LENGTH (mm)
0.3
0.2
0.1
0
0
5
10
15
20
25
DISTANCE FROM TIP OF COLEOPTILE TO NODE
(mm)

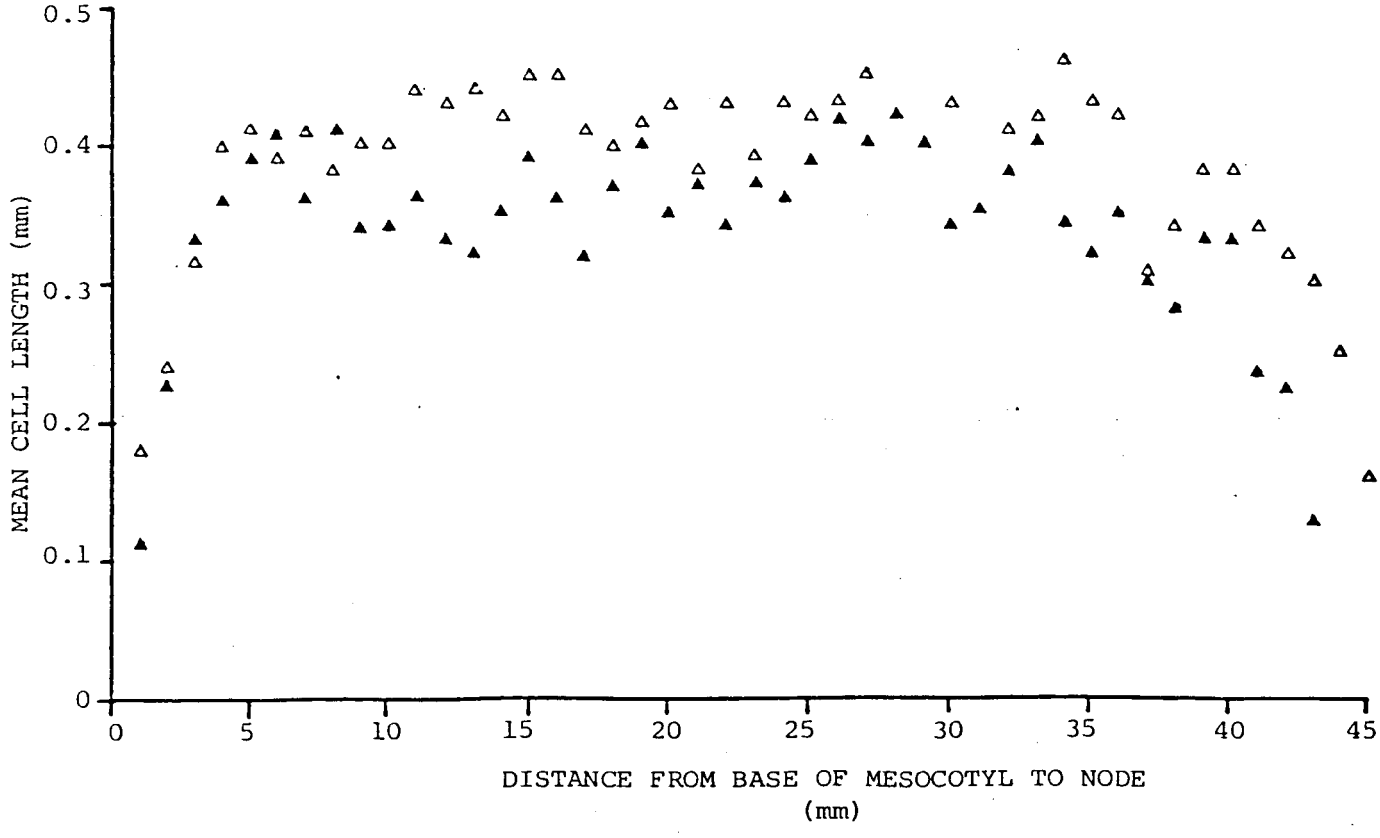

MEAN CELL LENGTH (mm)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

Figure 3.73: Distribution of cell length along the coleoptiles of 87 h old light-treated plants as modified by GA_3 injected into the endosperm

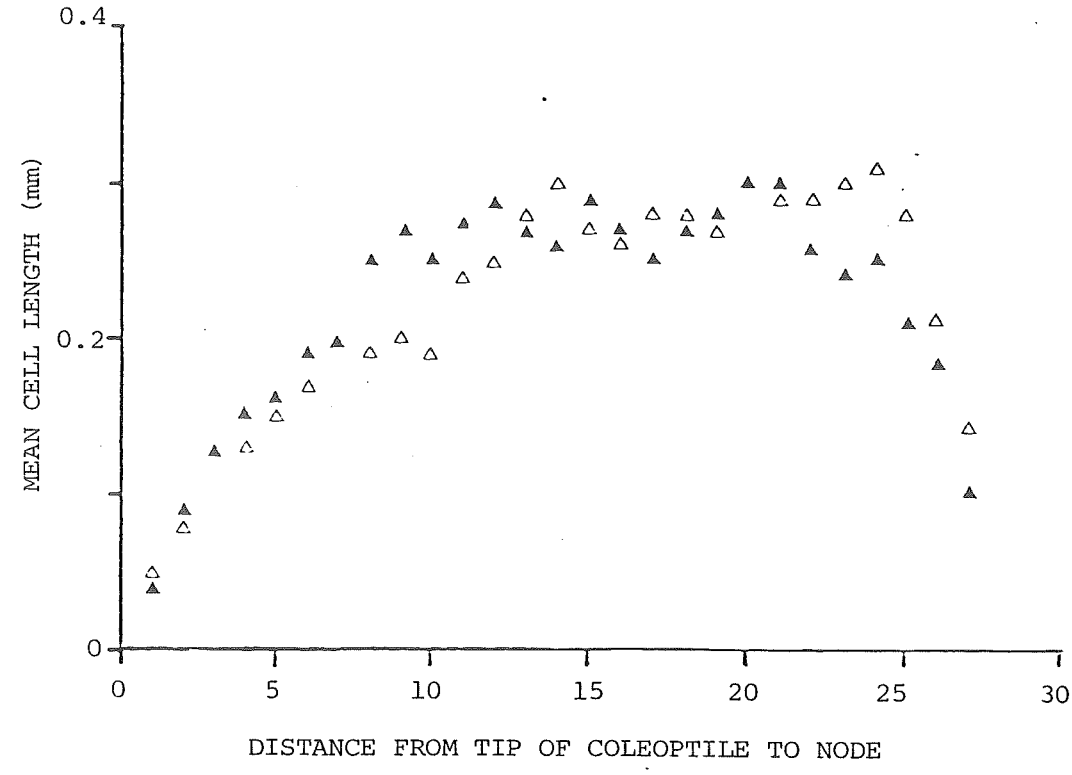
▲ Control

△ GA_3

Figure 3.74: Distribution of cell length along the mesocotyls of 87 h old light-treated plants as modified by GA_3 injected into the endosperm

△. Control

▲ GA_3


0.4
0.2
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
DISTANCE FROM TIP OF COLEOPTILE TO NODE

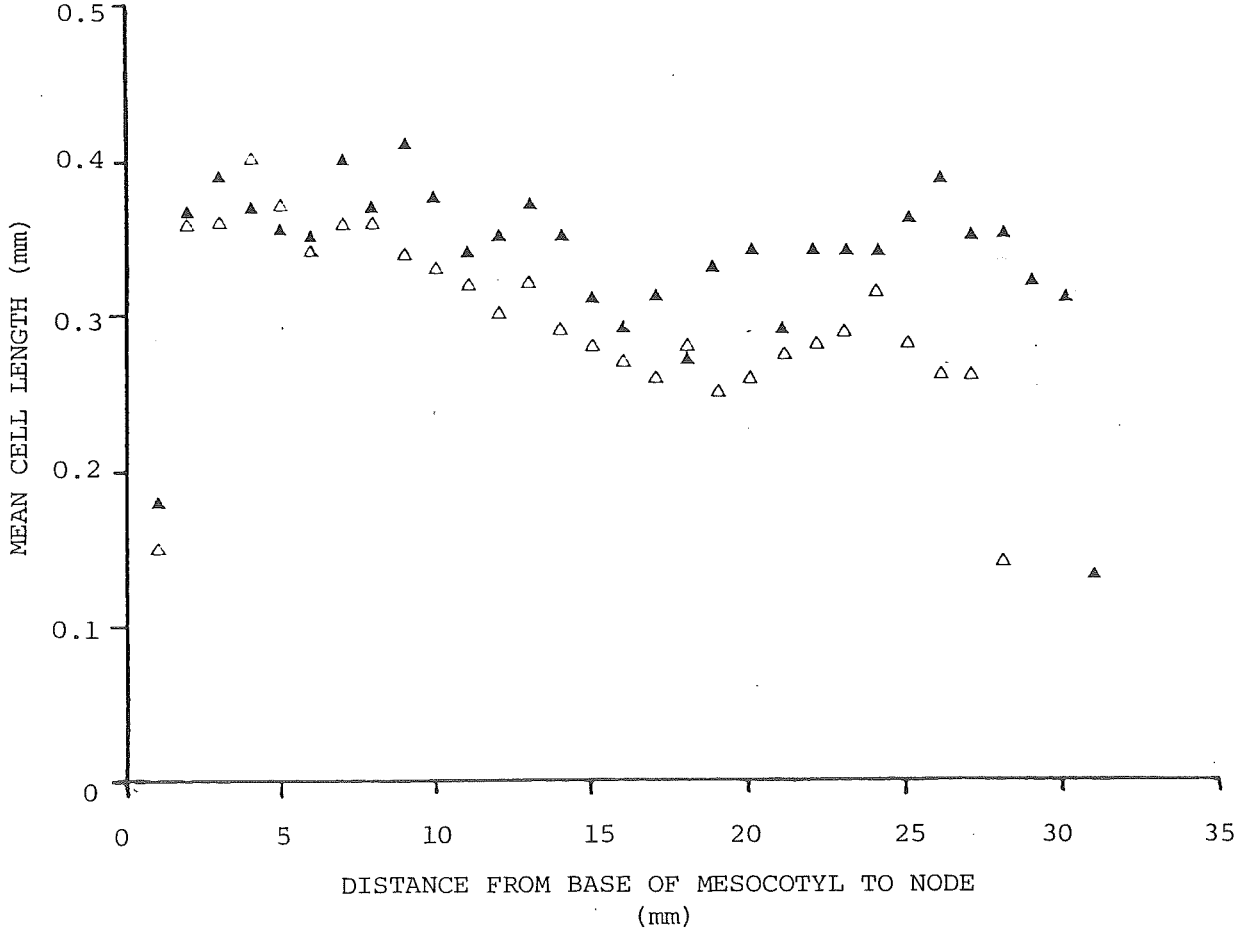

0.5
0.4
0.3
0.2
0.1
0
MEAN CELL LENGTH (mm)
0
5
10
15
20
25
30
35
DISTANCE FROM BASE OF MESOCOTYL TO NODE
(mm)

Table 3.27: Mean cell number per column in coleoptiles and mesocotyls of 87 h old dark-grown plants as modified by GA_3 injected into the endosperm

Seedling Treatment	Mean cell number per column	
	Coleoptile	Mesocotyl
Control (without GA_3)	160 ± 25	133 ± 5
GA_3	157 ± 24	124 ± 16

This effect was the result of increased mesocotyl cell extension. Cell division was not affected (Table 3.28, Student's t test, $P < 0.05$). Cells in the basal 8 mm of the mesocotyl did not respond to GA_3, but, elsewhere in the mesocotyl, extension of all cells was promoted and by approximately similar amounts.

Table 3.28: Mean cell number per column in coleoptiles and mesocotyls of 87 h old light-treated plants as modified by GA_3 injected into the endosperm

Seedling Treatment	Mean cell number per column	
	Coleoptile	Mesocotyl
Control (without GA_3)	148 ± 11	99 ± 14
GA_3	151 ± 22	97 ± 5

4.0 DISCUSSION

4.1 SEEDLING MORPHOLOGY AND GROWTH REGULATORS

The pattern of extension growth of etiolated oat seedlings, cv. 'Terra', reported here is similar to that described by other workers (Schneider, 1941; Weintraub and Price, 1947; Thomson, 1954; Mer, 1951, 1969). Considerable differences in final mesocotyl and coleoptile length can exist between cultivars of Avena. For example, under identical conditions to those used in this investigation, the mesocotyl and coleoptile of cv. 'Brighton' oats grew to 100 mm and 75 mm, respectively (Jackson and McWha 1976), which compares with the values reported here of 65 mm for the mesocotyl and approximately 70 mm for the coleoptile.

The inhibition of both coleoptile and mesocotyl extension when seedlings were grown in non-ventilated containers is contrary to the response reported by Mer and Richards (1950) following a similar experiment. They found mesocotyl elongation was promoted, while that of coleoptile was unaffected. When seedlings were treated with CO_2, extension growth of the mesocotyl was promoted and coleoptile elongation was inhibited (Mer and Richards, 1950; Mer, 1957; Mer and Causton, 1967). Again this is contrary to the results obtained in this study, where added CO_2 was found to inhibit both coleoptile and mesocotyl elongation. These differences could possibly be accounted for by the higher concentration of CO_2 employed by Mer and co-workers (5% in air, cf 1.5% and 3.0% used here). And also by the fact that in Mer's studies, CO_2 was administered during the first 3 d after planting, whereas in this investigation treatment was begun 24 h after planting and continued through to the 7th day. However, such an explanation cannot account for the difference in response observed when seedlings were incubated in closed containers without added CO_2, since, in both Mer and Richard's study and the present investigation, the containers remained sealed from the commencement of the experiment.

The histological studies showed that in the period from 63 h to 87 h after planting, the etiolated coleoptile

grew predominantly by elongation of existing cells, and by a small increase in cell numbers. This is consistent with the findings of Thomson (1954) and Mer and Causton (1967). The mean number of cells per column found in the 87 h old coleoptile (160 - 170), is comparable to the figures of 177 (Thomson, 1954) and 175 - 220 (Mer and Causton, 1967), estimated in 96 h old dark-grown coleoptiles. Both these workers have shown that cell division in the coleoptile persists for at least 4 d, although there is doubt as to when it finally ceases (Mer and Causton, 1967). During the 24 h period studied here elongation was approximately uniform along the length of the coleoptile, and this corresponds with the pattern that is evident in the data of Mer and Causton (1967)(also in dark-grown Avena), and in Avena coleoptiles which were exposed to diffuse light during the first day after planting (Avery and Burkholder, 1936).

It was shown that elongation of the etiolated Avena mesocotyl between 63 and 87 h after planting, was the result of a 35% increase in cell numbers and the extension of these new cells, as well as from elongation of the more apical existing cells. This growth pattern has previously been described for the Avena mesocotyl (Thomson, 1954; Mer and Causton, 1967). The majority of the cells in the basal half of the mesocotyl had achieved their final length (Mer and Causton, 1967) by the beginning of the experimental period and thus did not elongate further in the interval studied. Maturation in the mesocotyl occurs progressively from base to apex since new cells are produced by an intercalary meristem situated immediately below the coleoptilar node (Mer and Causton, 1967). The new cells remain meristematic until a length of about 0.12 mm is reached (Causton and Mer, 1966). According to these workers, cell production peaks on the second day after germination and ceases about the fourth day, but the length of the mesocotyl continues to increase for approximately a day, due to the elongation of cells produced just prior to the cessation of division. Mer and Causton (1967) reported the mean final length of cells

in the mesocotyl to be 0.5 mm, which is slightly more than 0.35 - 0.40 mm observed in this study.

The mean number of cells found in the cortical files of mesocotyls, 87 h after planting (126-133), is similar to the figure of 134 reported by Thomson (1954) in 72 h old etiolated oat mesocotyls. Interpolation of the data of Causton and Mer (1966)(their Fig. 2), gives a value of approximately 135 cells at age 87 h. The slight differences in cell numbers and cell lengths in the coleoptiles and mesocotyls in this study and those reported by other workers, could be due to differences in the growth conditions employed and the different cultivars of oats used (both Thomson and Mer used 'Victory' oats while the 'Terra' cultivar was employed here). In addition, exposure to the 'safelights' during selection of the plants at the beginning of the histological studies could have partially inhibited cell division in the mesocotyl (Mer, 1966).

The green 'safelights' used in this investigation were shown to inhibit mesocotyl elongation by up to 16%, depending upon the duration of exposure. This is consistent with the so-called first, low-irradiance response described by Blaauw et al. (1968), and Mandoli and Briggs (1978/79, 1979/80). According to the latter workers, all known 'safelights' induce this response in Avena. That green light is not morphogenetically inactive has been known for some time (Goodwin, 1941; Schneider, 1941; Weintraub and McAlister, 1942; Goodwin and Owens, 1951; Huisinga, 1964). Mer (1966) however appears to be the first to appreciate the effect that even a dim green 'safelight' can have on the growth of the etiolated oat plant. It has been claimed (Mandoli and Briggs, 1979/80), that uncontrolled 'safelight' conditions have been a major variable in most reports on Avena seedling growth.

Unfortunately, for certain manipulations (e.g. injection into the endosperm, as in this study) the use of a 'safelight' is necessary; in which case, at best, exposure of the seedlings can be carefully standardised. Here, 'safelight' exposures were kept to between 5 and 10 minutes,

where the response appeared to be saturated Fig. 3.3 (cf Blaauw et al., 1968). Shorter exposure times were avoided since slight variations in the length of exposure markedly affected the degree of mesocotyl inhibition. It is therefore important that when 'safelights' are used, a dose-response curve is first determined, so that the 'optimum' exposure time can be chosen to reduce variability between exposed plants. Variable duration of exposure could conceivably result in apparent treatment effects in certain experiments.

All 'safelight' exposures, however, modify the growth of the 'etiolated' Avena seedlings, and these effects must be considered when experimental results are interpreted. Recently, Iino and Carr(1981) have described a system using an infra-red scope and infra-red radiation which they claim is truly 'physiologically safe'. This procedure would appear to hold great promise for future work on light-sensitive plant systems.

The response of seedlings to red light, which inhibited final mesocotyl length by approximately 50% and promoted the rate of coleoptile extension (although reducing its ultimate length), is similar to the response reported elsewhere for illuminated Avena seedlings (Thomson, 1954; Mer and Causton, 1967; Blaauw et al., 1968; Mandoli and Briggs, 1979/80). This corresponds to the second-phase, light response described by Blaauw et al. (1968), which is apparently phytochrome-mediated (Blaauw et al., 1968; Mandoli and Briggs, 1979/80). Although no alteration in the growth rate of coleoptile was apparent in the 24h period immediately after red light illumination, the cell studies established that: in the upper half of the coleoptile, cells tended to be longer than in dark-grown coleoptiles; while in the lower half, cells were shorter. Cell numbers were unaffected. Thomson (1954) and Mer and Causton (1967) have both studied the longer-term effects of light treatment on Avena coleoptile extension. Mer and Causton found a 5 min exposure to dim red light, given 3 d after planting, had no effect on cell division (as was found in this study), and resulted in a temporary stimulation of cell elongation. However final cell length

in the illuminated coleoptiles (achieved after 7 d), was less than in the controls. On the other hand, Thomson (1954), who used a much higher dosage of light, found cell division was slightly inhibited, but the effect on cell elongation was similar to that reported by Mer and Causton.

According to Mer (1969), the temporary stimulation of cell elongation is probably not a direct effect but is the result of an increase in the availability of nitrogenous substances to the coleoptile, due to photo-inhibition of mesocotyl elongation. He believes that the growth of the mesocotyl limits the transfer of nutrients from the endosperm to the coleoptile. Support for this theory comes from the observations of Mer (1959) and Mer, Dixon, Diamond and Drake (1963), that nitrogenous metabolites supplied in small amounts increase the length of the coleoptile. Other evidence, however, does not favour this 'starvation' theory. Supplying exogenous sucrose does not have a promotive effect on coleoptile growth. In fact, elongation of the coleoptile is inhibited and mesocotyl extension is promoted (Mer, 1959; Mer and Causton, 1967).

Photo-inhibition of mesocotyl elongation has generally been interpreted in terms of a decrease in available auxin, and the 'classical' hypothesis (e.g. Inge and Loomis, 1937) holds that light treatment reduces the supply of auxin from the coleoptile tip to the mesocotyl. In this study, it was found that exogenous IAA applied to the coleoptile tip could reverse, almost completely, the inhibition of mesocotyl extension resulting from light treatment. These findings are in contrast to those of Schneider (1941), who found IAA in lanolin paste applied to the coleoptile tip, overcame only 30% of the light inhibition of intact Avena mesocotyls. As in this investigation, Schneider treated his plants 63 h after planting. However mesocotyl length was determined after a further 81 h incubation, compared to 24 h in this study. Although Schneider used a higher concentration of IAA than here (0.1% w/w cf. approximately 0.04% w/v), only a small amount of paste was applied to the coleoptile tip whereas large agar cylinders (60 mm^3) were employed in these

experiments. Furthermore, release of IAA from lanolin is poor compared to agar (Patrick and Woolley, 1973). Together these factors would suggest that Schneider's plants were not exposed to high IAA levels throughout the duration of his experiments, and this would account for the lower amount of promotion observed. In Zea, IAA applied in lanolin paste to the coleoptile tip has been reported to overcome almost fully, photo-inhibition of mesocotyl extension (Inge and Loomis, 1937), and complete reversal has been reported in heat-treated seedlings (van Overbeek, 1936).

The reduction in the rate of cell division in the mesocotyl and the limited extension of certain cells following illumination found in this study, is similar to that reported by Mer and Causton (1967), also in Avena. According to these researchers, the degree by which cell elongation is affected depends on the length of the cell at the time of light exposure. They found cells that were approximately 0.12 mm long when exposed, were the most sensitive, while cells longer or shorter than this were inhibited to lesser degrees. Thus, a group of short cells flanked by longer ones was apparent in light-treated mesocotyls after a period of incubation (Mer and Causton, 1967). In this study, a similar group of short cells was observed in mesocotyls that had been illuminated, but the cells most affected were 0.20 - 0.21 long at the time of exposure. Thomson (1954) believes that cells in the process of division are probably not susceptible to the effects of light. Heat treatment has been shown to have a similar effect to light on cell division and elongation in Avena mesocotyls (Mer and Causton, 1967).

The above observations are not consistent with the 'classical' auxin hypothesis, that light regulates mesocotyl elongation by controlling the supply of auxin from the coleoptile. As Mer (1969) has noted, a change in the amount of auxin diffusing into the mesocotyl, cannot account for the fact that, in Avena at least, short cells are found sandwiched between longer ones in the light-inhibited mesocotyl i.e. some cells are inhibited more than others. It seems more likely that the sensitivity of certain cells to auxin is altered by light (Mer, 1969; Osborne 1978/79).

Thus when excess IAA is applied, the inhibited cells grow to their normal length. The change in the sensitivity of cells might involve a change in the enzymes responsible for IAA catabolism or, as postulated by Bandurski's group, a change in the enzymes that make and hydrolyse IAA-conjugates in the shoot (Bandurski et al., 1977; Bandurski, 1980).

A change in cell sensitivity would account for many of the inconsistencies highlighted in the 'Introduction', with respect to the control of mesocotyl elongation. Notably, these are: that direct illumination of the cells near the coleoptilar node is more effective in inhibiting mesocotyl elongation in Avena than illumination of the coleoptile tip (Araki and Hamada, 1937; Mer, 1969); that the elongation of isolated Avena mesocotyl segments can be inhibited directly by light (Goodwin, 1941); and that mesocotyls of oat plants which have been briefly illuminated contain at least as much ether-extractable auxin as mesocotyls from control plants (Dattaray and Mer, 1964).

The evidence supports Mer's view that the growth of the Avena mesocotyl is independent of a supply of auxin from the coleoptile, since removal of the coleoptile tip (Mer, 1951), or the whole coleoptile (Mer, 1972), does not inhibit mesocotyl extension. There is evidence that in Zea, auxin from the coleoptile is involved in regulating mesocotyl extension. This is discussed in detail below.

The effectiveness of application of IAA, in agar to the coleoptile tip, or by injection into the grain, cannot be compared without first knowing what proportion enters the shoot. Shen-Miller, Cooper and Gordon (1969) applied IAA-^{14}C in agar blocks to the tips of intact Avena coleoptiles. The concentration used was 3.25×10^{-5} M, which is close to the lowest application rate of IAA in agar used in this investigation (2.5×10^{-5}M). They found 3.1 ng was taken up by the plant in 2 h. Since the uptake of IAA by the oat coleoptile is proportional to the concentration of growth substance in the agar block (Went and Thimann, 1937), the expected uptake from a 2.5×10^{-5} M IAA block in 2 h would be approximately 2.4 ng.

The IAA-labelling experiments provide a measure of the amount of IAA entering the shoot following injection into the endosperm. The 200 Bq experiment corresponds to the amount of IAA applied when 4 µl of 2.5 x 10^{-5} M IAA was injected. In 3 h, 15 Bq of radioactivity moved into the shoot (scutellum excluded) and about half of this was IAA-^{14}C. Since the specific activity of the IAA-^{14}C was 11.28 Bq.ng^{-1}, this represnets about 0.7 ng IAA. After allowing for differences in transport times, these figures indicate that almost 6 times more IAA enters the shoot when applied to the tip than when a solution of an equivalent concentration is applied to the endosperm.

Once in the shoot, IAA appears to be metabolised at a similar rate, irrespective of the method of application. Shen-Miller et al. (1969) found IAA-^{14}C was rapidly metabolised in the tip, after application to the apex of the shoot; but they gave no indication of what proportion of the total IAA taken up this comprised. However, IAA-^{14}C in agar, applied to the apical end of Avena coleoptile and mesocotyl segments (Malloch and Osborne, 1976), was metabolised at rates very similar to the rate of IAA metabolism observed in this investigation, when IAA-^{14}C was injected into the endosperm.

Comparison of the effect of IAA applied to the coleoptile tip, and to the grain, is complicated further by the fact that IAA translocation from the endosperm declines markedly after the first 1-3 h. This, combined with the rapid rate of metabolism of IAA in the shoot, means that high IAA levels probably persisted in the tissue for less than 6 h after injection. On the other hand, transport of IAA from the agar capsule is likely to be continuous throughout the experiment (24 h). The agar blocks used in this study were large (60 mm^3), and at the rate of transport projected earlier, a less than 10% alteration in the concentration of IAA in the donor block would have occurred. As noted above, the rate of uptake of IAA from agar blocks is proportional to its concentration in the block (Went and Thimann, 1937). Because of the increasing concentration of IAA within the

shoot, the rate of uptake may have slowed somewhat. However, even after 24 h, a 10 fold concentration differential would still exist between the donor block and the shoot tissue, so any decline in uptake by the shoot would have been only slight. These approximations indicate that any difference in the effectiveness of IAA applied to the coleoptile tip or to the grain probably relates to the rate of IAA entry to the tissue and the duration of the IAA supply, and not to any inherent difference in the mode of action of IAA applied by either manner.

With the highest IAA injection amount, extension of the light-inhibited mesocotyl was promoted, but elongation of the coleoptile was unaffected. Application of IAA to the coleoptile tip, which produced similar mesocotyl promotion, also stimulated elongation of the coleoptile. This different response probably merely reflects the amount of IAA entering the coleoptile in each case. The radio-tracer experiments indicate that over the period in which free IAA-^{14}C persisted in the shoot following application to the grain, the concentration of IAA was approximately half of that in the mesocotyl. Mousdale (quoted by Malloch and Osborne, 1976) has shown that the endogenous IAA content of the Avena coleoptile is double that of the mesocotyl, and presumably this indicates a differing sensitivity of the cells to IAA. Certainly, isolated Avena coleoptile segments are less sensitive to applied IAA than mesocotyl segments (Nitsch, 1956; Jackson and McWha, 1976). Thus, the quantity of IAA reaching the coleoptile from the grain, most likely was insufficient to promote extension growth.

A rather surprising result was that while application of IAA to the coleoptile tip promoted coleoptile extension, it inhibited elongation of the primary leaves. This was the case in both light-treated and dark-grown seedlings. Similar effects have been observed in coleoptiles of light-treated Avena (Went, 1935) and heat-treated Zea plants (van Overbeek, 1936), following application of IAA paste to the apex. According to Went (1935), growth of the primary

leaves is probably inhibited due to competition for nutrients from the grain. He showed that the effect is not due to IAA itself, since when the coleoptile is excised and IAA applied to the cut stump, no inhibition of leaf growth occurs.

Another unusual effect was the apparent reversal of the normal geotropic response in both dark-grown and illuminated Avena shoots, following application of IAA in agar to the coleoptile tip. No such response was reported by Went (1935), when IAA in lanolin paste was applied to the tips of intact Avena coleoptiles, or by van Overbeek (1936) or Inge and Loomis (1937), when similar applications were made to heat-treated and light-treated Zea seedlings respectively. Nor is this phenomenon referred to in major reviews on geotropism (Wilkins, 1966, 1979; Juniper, 1976; Reinhold, 1978; Firn and Digby, 1980; Firn, Digby and Hall 1981). Since shoots grew directly downward when treated with the highest IAA concentration, the observed effect appears to involve a reversal of the geotropic response, rather than simply a loss of sensitivity to gravity as might be expected if supra-optimal concentrations of IAA were having a toxic effect.

Intact mesocotyls were more responsive to GA_3 injected into the endosperm than IAA. This was the case for both light-treated and dark-grown plants. Similarly, GA_3 partly reverses photo-inhibition of the mesocotyl in Avena when applied via the roots, whereas IAA has no effect (Jackson, unpublished). Furthermore, Mujica (1976) has reported that GA_3 applied to the roots is more effective than IAA in reversing the inhibition of mesocotyl extension caused by heat treatment of etiolated maize seedlings. The greater effect of exogenous GA_3 may be due to a slower rate of metabolism of GA_3 in the shoot and endosperm.

GA_3 does not appear to act indirectly by stimulating starch hydrolysis, since Mujica (1976) also observed a promotive effect in maize seedlings grown from isolated embryos and cultured on nutrient agar. Furthermore, the elongation of isolated Avena mesocotyl segments is promoted by gibberellin (Milborrow, 1966). The histological studies here indicated that GA_3 stimulated cell elongation while cell division was unaffected.

ABA was a highly effective inhibitor of coleoptile and mesocotyl extension in both etiolated and light-treated

plants, and at the highest application rate tested (2.64 μg. $grain^{-1}$), elongation of the mesocotyl and coleoptile was almost totally suppressed. The inhibitory effect of ABA on isolated mesocotyl and coleoptile segments is well known, as these tissues are commonly used as bioassays for growth inhibitors (Nitsch, 1956; Barlow, Hancock and Lacy, 1961; Milborrow, 1966). However, ABA at low concentrations can also promote extension of coleoptile and mesocotyl segments (McWha and Jackson, 1976); and ABA promotion of mesocotyl growth in etiolated rice seedlings (Takahashi, 1972, 1973), and in maize seedlings following heat treatment (Mujica, 1976), has been reported.

Kinetin was found to have no effect on the elongation of the shoot and roots when injected into the endosperm. This is surprising, since treatment of kinetin has been shown to increase the endogenous IAA content of seeds of *Phaseolus vulgaris*, *Zea mays* and *Pinus silvestris*, as well as the IAA levels of all parts of the young bean plant (Saleh and Hemberg, 1980). Further, Jordan and Skoog (1971) found more auxin is released into agar from *Avena* coleoptile tips treated with synthetic cytokinin than from untreated tips. However in whole plant studies, these workers found application of the synthetic cytokinin, 6-[3-methyl-2-butenylamino] purine (2iP), to the coleoptile tip of light-treated *Avena* seedlings, had no effect on coleoptile extension. The effect of derooting, which inhibited elongation of the coleoptile, could be reversed by application of 2iP, either to the coleoptile tip or to the cut base of the roots.

Application of a range of different plant growth regulators had no effect on extension growth of the roots. The radiotracer studies showed that radioactivity was translocated into the roots following injection of IAA-^{14}C to the endosperm. Since it was concluded that transport of applied radioactivity out of the grain was non-selective (discussed below), the other growth regulators were probably similarly transported into the roots. Inhibition of root growth by ABA might have been expected, since the effect on shoot extension was so dramatic, and ABA applied to the roots of oat seedlings does inhibit elongation of the roots (Jackson, unpublished). For ABA, at least, these results tend to suggest that the hormone was metabolised *en route*.

4.2 IAA-2-^{14}C STABILITY

IAA-^{14}C was found to be stable when incubated with 95% ethanolic extracts of Avena shoots and grains, and when evaporated to dryness under reduced pressure; but considerable destruction of IAA-^{14}C was observed on silica gel TLC plates. Mann and Jaworski (1970) have previously reported almost 50% destruction of IAA incubated for 3 h with 80% ethanolic extracts of soyabean leaves. In this investigation however, 95% ethanol was used as the extracting solvent (and this included an initial extraction in absolute ethanol), so it is likely that the enzymes responsible for IAA oxidation were inactivated (Nowacki and Bandurski, 1980).

Erratic breakdown of IAA-^{14}C added to methanol extracts of Phaseolus shoots was observed by McDougall (1978). However, he was unable to determine whether destruction was occurring during incubation in the extract or in one of the later purification stages. Breakdown during rotary film evaporation was eliminated, since IAA-^{14}C was stable even when methanol or ethereal solutions were subjected to extended periods of evaporation. Similarly, in this study, no destruction of IAA-^{14}C was found when 95% ethanol solutions were taken to dryness.

In contrast, Mann and Jaworski (1970) and Iino, Yu and Carr (1980) have reported substantial loss of IAA when solutions are dried. Mann and Jaworski concluded some IAA sublimed *in vacuo*, but according to Iino and co-workers, it is more likely that some of the decomposition products of IAA formed during evaporation are volatile. These workers showed that considerable decomposition of IAA occurred when a range of organic solutions of IAA were taken to dryness, even under a stream of nitrogen at normal atmospheric pressure. The breakdown was apparently due to impurities in the solvents and could be eliminated by treating the solvents with butylated hydroxytoluene (BHT, an antioxidant), washing them with water, and passing the solvent through cotton wool (Iino *et al.*, 1980).

The lack of destruction of IAA when solutions were taken completely to dryness, in this study and in that of McDougall (1978), presumably indicates the particular impurities responsible for decomposition were absent from the solvents. However, since Iino *et al.* (1980) noted the effect varied between different batches of solvent, some

breakdown of IAA may have occurred in later experiments in which solvent from other batches was used. This is unlikely because control solutions of authentic IAA-^{14}C in 95% ethanol were carried through the purification procedure in every experiment, and in no instance were any breakdown products apparent following thin-film evaporation. However, purity was checked only by TLC. Since total recovery was not quantified, the possibility remains that IAA or some volatile decomposition products were lost during evaporation. Such losses would not be reflected in the chromatogram.

IAA-^{14}C was found to be rapidly broken down on silica gel TLC plates, and under normal laboratory conditions only 25% of the applied IAA was recovered after 1 h. This decomposition first became apparent following short delays in sample application and chromatogram development. Destruction of IAA during application of IAA solution to the origin of silica gel-impregnated, chromatography sheets has been previously reported (Mann and Jaworski, 1970); but what was surprising here was the speed with which IAA was lost (25% in 15 min). McDougall and Hillman (1978) have also noted that IAA is broken down on silica gel plates prior to development and that breakdown is accelerated by light and time. However, they gave no indication of the rate or extent of decomposition. Mann and Jaworski (1970) found almost complete loss of IAA from developed chromatographs stored overnight, presumably in the dark, and Sagi (1969) reported 50% IAA loss from silica gel plates over a similar period in darkness.

Breakdown of IAA on silica gel probably involves oxidation, and losses have been reduced considerably by applying antioxidant to the origin prior to sample loading (Mann and Jaworski, 1970). At least nine decomposition products of IAA-^{14}C were observed here, but these were not identified. In fact, none of the breakdown products have been identified in any of the studies to date. Consistent with the results of Sagi (1969) and Mann and Jaworski (1970), these products (with the exception of a minor spot at the origin) did not react with Salkowski reagent. This fact has

probably prevented many workers from noting that IAA destruction was occurring during TLC.

Breakdown of IAA is a result of incubation on the silica gel, and the chromatography solvent itself causes little or no destruction. This is in agreement with the findings of Mann and Jaworski, (1970) and Iino et al. (1980). Moreover, breakdown appears to be a property specifically of silica gel, since little decomposition of IAA occurs if cellulose (Sagi, 1969; Iino et al., 1980) or polyamide gel (Iino et al., 1980) is used as the solid support. Therefore, it is doubtful whether IAA is oxidised simply because of the large surface area presented by the silica gel, as was suggested by Sagi (1969).

Breakdown was accelerated greatly by light. The light and silica gel may act synergistically, since the loss of radioactivity when Iino et al. (1980) exposed IAA-1-^{14}C dried on glass to laboratory light for 3 h, was only 13%, compared to the 75% loss observed here from TLC plates in 1h. However, because Iino and co-workers measured only decarboxylation, their figure may not accurately reflect total IAA decomposition.

In this investigation, it was shown that substantial loss of IAA-^{14}C occurs on silica gel TLC plates (25% in 15 min), in what most researchers would probably consider a 'safe' interval from the beginning of sample application. These losses could result in serious errors in the estimation of endogenous IAA levels in plant extracts, or lead to the wrongful identification of IAA 'metabolites' in such extracts or in radio-tracer studies.

Normal laboratory practices have probably led to considerable inadvertent destruction of IAA during application to silica gel TLC plates. In instances where a number of samples are applied to the same plate, or where large sample volumes are involved, the delay in development of the plate could result in substantial decomposition of the first-applied IAA. This problem may be exacerbated with plant extracts, where contaminating material can create difficulties with application of the sample to the chromatograph and thus

further delay development. The use of an internal standard of radioactive IAA enables correction for such losses (Knegt and Bruisnsma, 1973; Bandurski and Schulze, 1974; Little, Heald and Browning, 1978; Sweetser and Swartzfager, 1978; Iino et al., 1980).

In quantitative work, destruction of IAA on the TLC plate after development is probably potentially more serious. This is particularly so where silica gel TLC is the final purification step prior to estimation of IAA levels. In such instances, IAA is commonly measured either directly by chromogenic sprays (Zimmermann and Rudiger, 1976), or after elution of the appropriate zone from the plate and quantification by UV absorbance, colorimetry, (Percival and Bandurski, 1976; Epstein et al., 1980), or fluorimetry (Iino et al., 1980).

Generally, IAA-^{14}C standards are also chromatographed either in parallel with the sample, or as an internal standard. The standard spot itself, or an aliquot of the eluted IAA zone, is then counted by LSA to determine the recovery of the labelled IAA to enable correction for losses of IAA during TLC.

This procedure, however, may not provide an accurate estimation of IAA decomposition on the plate after development since, if IAA-2-^{14}C is used as the standard, the label is retained in the decomposition products. Thus LSA will not indicate that breakdown has occurred after development and prior to elution. Since the 'cold' IAA decomposition products are not detectable by the assays described above (Sagi, 1969; Mann and Jaworski, 1970; Iino et al., 1980), the IAA-^{14}C standard will underestimate the actual losses of IAA on the silica gel plate.

Photodecomposition of IAA in aqueous solution (Epstein and Lavee, 1975), and when dry (Iino et al., 1980), involves decarboxylation. Some or all of the degradation of IAA on silica gel plates may also involve loss of the 1-carbon, and therefore IAA-1-^{14}C might be a more useful standard for estimating IAA losses during TLC. However, this has not been established. The benefits of using 1-labelled IAA

as an internal standard during extraction and purification of IAA-containing extracts, have previously been discussed by Iino et al. (1980).

The data presented here show that up to 50% loss of IAA could occur during TLC on silica gel under normal circumstances, if 10 minutes is allowed for sample application, and a further 15 minutes for drying of the plate after development prior to IAA estimation. Such losses are not considered to be unrealistic (see Bandurski and Schulze, 1974) and could result in serious underestimation of IAA levels in plant extracts.

4.3 IAA-^{14}C TRACER STUDIES

The multiple solvent extraction system proved unsatisfactory for the extraction of IAA-^{14}C and its derivatives from intact Avena grains. Thus material that was difficult to extract was misidentified as 'bound' IAA. A similar mistake was probably made by Hemberg (1955) who, when extracting IAA from intact Zea kernels with ether, may have confused 'difficultly extractable' IAA with bound IAA (Bandurski, pers comm.).

The differential extraction system employed here has been used previously to extract IAA and its metabolites from whole pea stem segments (Davies, 1976), Nicotiana petiole slices (Liu, Gruenert and Knight, 1978) and leaf tissues of woody plants (Riov, Goren and Dror, 1981). Although Avena grains are larger than the pea stem segments of Davies, the preliminary experiment (section 3.3.2.1) appeared to indicate that differentiation of the fractions could be achieved by increasing the incubation times in each solvent, for the grains. However, it was found later that some of the 'X' and 'Y' metabolite remained in the grain after 95% ethanol extraction, and were extracted by water. These compounds are clearly less soluble in 95% ethanol than IAA, since comparison with other extraction methods shows that all the free IAA was extracted by 95% ethanol, even from whole grains. Because of the low solubility of fractions

'X' and 'Y', the amount of radioactivity obtained by successive extractions in 95% ethanol in the preliminary experiment appeared insignificant, relative to the total amount of 95% ethanol-extractable radioactivity and the radioactivity obtained when the grains were transferred to water.

Furthermore, the preliminary experiment involved grains that had been incubated for 3 h after injection, whereas the experiments in which the proportions of free, bound, 'X' and 'Y' were determined, were incubated for 6 h. Thus 'X' and 'Y' would have formed a lower proportion of the total radioactivity in the preliminary experiment, and thus be less apparent.

The 95% ethanol-based differential extraction system appears to be satisfactory when grains are macerated prior to extraction, particularly if care is taken to avoid transfer of particulate matter during decanting. However, the best system would probably be to terminate the experiments by finely grinding the grains in 50% acetone, followed by fractional precipitation in a manner similar to Percival and Bandurski (1976).

It was found that IAA-^{14}C was rapidly metabolised in the grain, irrespective of the amount applied. Considering the differences in the quantities injected, the proportion of applied IAA metabolised in each experiment in 6 h was very similar (about 80-88% of the total radioactivity in the grain). The metabolites were formed in the seedling and were not artefacts of extraction. They increased in amount with longer incubation times in the plant, and were not formed when IAA-^{14}C was incubated with grains in 95% ethanol, under conditions similar to those used for extraction of the radioactivity from injected grains.

Analysis of the ^{14}C-labelled products 6 h after injection of 2 kBq IAA-^{14}C, indicated that about 40% of the metabolised IAA consisted of acetone-precipitable material associated with carbohydrate and ninhydrin-positive substances. The low amount of acetone-precipitable radioactivity occurring in 95% ethanolic extracts of macerated grains, indicates this material is probably insoluble in high

concentrations of ethanol, as well as acetone. The radioactivity, carbohydrate and ninhydrin-positive material also purified together through a variety of chromatographic systems, which suggests that the radioactivity was co-valently linked to substances in the complex.

Similar amounts of radioactivity were obtained from the metabolite fraction by mild alkaline hydrolysis or acetone-precipitation. This suggested that the acetone-precipitable material was also the hydrolysable, IAA-containing material. The IAA was definitely released from the carbohydrate/protein complex (Figs. 3.29 and 3.30), and the amount liberated was too large to have come from the 'X' metabolite fraction. Since inadvertent hydrolysis of alkali-labile IAA-conjugates may occur during extraction and purification (Ueda and Bandurski, 1969; Bandurski and Schulze, 1974; Nowacki and Bandurski, 1980) it is possible that the proportion of conjugated IAA in the grain may have been higher than estimated here.

A small proportion of the radioactivity released by mild hydrolysis consisted of substances other than IAA. These were ether-insoluble and were apparent when hydrolysed extracts were chromatographed without purification, but not when the products were first extracted into ether. This presumably accounts for the small discrepancy between the amount of radioactivity that was precipitated and the amount that was determined by extraction into ether after 1N NaOH-hydrolysis.

A small amount of hydrolysable and precipitable radioactivity was present in the 95% ethanol extracts of macerated grains. This material was probably insoluble in 95% ethanol, but was washed out of the test tube when the solutions were decanted following centrifugation. During extraction, the ethanol solution was changed three times, and it was noticed that some particulate matter remained suspended in the supernatant.

The ease with which IAA-^{14}C was released from the 'bound' complex suggests that it was probably esterified. IAA-ester is usually characterised simply by its ready hydrolysis in dilute base at room temperature (Ueda and

Bandurski, 1969; Bandurski and Schulze, 1977; Hall and Bandurski, 1978; Purves, 1978; Iino and Carr, 1982a), whereas peptide-bound IAA is only liberated by strong base at elevated temperatures (Bandurski and Schulze, 1977; Purves, 1978). Radioactivity was uniformly associated with the carbohydrate part of the complex, whereas the amount of ninhydrin-reactive substances varied considerably, suggesting that the IAA was linked to the sugar and, that peptide chains of different length and/or composition, were attached to the carbohydrate moiety. This would suggest a glycosidic (ester) linkage between the IAA and the carbohydrate. The endogenous IAA-glucoprotein ester in Avena, described by Percival and Bandurski (1976), showed considerable heterogeneity with respect to the size and composition of the side-chain, but nevertheless, ran as a discrete band during TLC.

An endogenous IAA-containing compound with properties similar to the labelled conjugate was also isolated from grains of dark-grown Avena seedlings, using a technique similar to that of Percival and Bandurski (1976). The production of IAA, and what appeared to be indole-3-acetamide (IAM), upon ammonolysis of the endogenous complex, indicates that IAA was bonded by an ester linkage. The labelled and the endogenous IAA-conjugates share a number of properties with the endogenous IAA-glucoprotein ester of Percival and Bandurski. They were soluble in 50% aqueous acetone, but were precipitated by high concentrations of acetone. Also, the compounds moved as a single band on TLC, were charrable with H_2SO_4 and fluoresced under UV; and in all cases, the IAA co-chromatographed with carbohydrate material and ninhydrin-positive substances. Furthermore, the polymer band was faintly yellow on TLC [as reported by Percival and Bandurski (1976)], which was attributed to the presence of phenolic substances co-valently linked to the IAA-ester complex (Percival and Bandurski, 1976). In this study, it was noted that this band became dark brown after exposure to air, consistent with colour changes found on oxidation of phenolics.

In these experiments, the IAA-conjugates ran to Rf 0.61-0.72 during TLC in chloroform/methanol/water (3:5:2)

(v/v/v), whereas Percival and Bandurski reported Rf 0.75 - 0.9 in the same solvent system. These differences could be accounted by the lower degree of purification employed in this study and to differences in the chromatography conditions. Only one carbohydrate-containing band was apparent on the chromatogram, and as purification was less rigorous than in Percival and Bandurski's study, it is unlikely that the IAA-glucoprotein would be lost during purification. However, since it was shown here that metabolite 'X' also co-chromatographed with the IAA-conjugate, and that ammonolysis of the labelled 'bound' extract produced predominantly IAA and only a small amount of putative IAM, the identification of both the endogenous and the labelled IAA conjugates is not equivocal.

The identity of metabolite 'X' is much less certain. This material was chromatographically similar to the IAA-conjugate, but was not precipitated by acetone. It accounted for about 35-37% of the metabolised IAA in the grain, 6 h after injection of 2 kBq IAA-^{14}C. After separation from the conjugated IAA by acetone-precipitation, the radioactivity continued to co-chromatograph with ninhydrin-positive material. It was not established whether carbohydrate also remained.

The apparent association between radioactivity and the ninhydrin-positive material, plus the fact that 'X' was soluble in 95% ethanol and 90% acetone, but insoluble in ether, suggests the metabolite could consist of IAA bound to a peptide (Davies, 1972). Minchin and Harmey (1975) found that IAA-^{14}C was converted to indole-3-acetyl asparate (IAAsp), via an unidentified intermediate, in barley endosperm halves incubated in IAA-^{14}C solution (the intermediate ran ahead of IAA during TLC in isopropanol/ammonia/water and therefore bears no relationship to any of the compounds formed in this study). Peptide-bound IAA should be hydrolysed completely within 3 h in 7 N NaOH at 100°C (Andreae and Good, 1955; Bandurski and Schulze, 1977). Some additional ether-soluble compounds did become apparent when the extracts were vigorously hydrolysed, but because

of losses of radioactivity during the hydrolytic treatment, it is unclear whether these new compounds were actually released by hydrolysis, or whether they were breakdown products of IAA or 'Y'. The bulk of the radioactivity remained in the aqueous phase. It is possible that IAA was released by hydrolysis, but was degraded to ether-insoluble products, and thus remained in the aqueous fraction. Since treatment with proteolytic enzyme failed to release any radiactivity from the complex, it seems unlikely that 'X' consists of peptide-bound IAA. Such enzyme treatment has been shown previously to release IAA-^{14}C from protein conjugates formed in pea stems (Morris, Briant and Thomson, 1969). The nature of 'X' therefore, is unclear. It may simply be a catabolite of IAA which co-chromatographs with the mild alkali-labile IAA - conjugate, and with the ninhydrin-positive material.

The labelled compound designated 'Y' was present at about half the level of 'X', in extracts from the 6 h harvest. Like 'X', it was soluble in 95% ethanol or acetone, and insoluble in ether. However it was not associated with carbohydrate, or ninhydrin-positive material, and was probably an IAA-^{14}C catabolite. This compound was not further characterised.

Because the proportion of conjugated IAA, and metabolites 'X' and 'Y', were studied only at the 6 h harvest time in the 2 kBq experiment, it is not known whether the relative amounts formed were dependent on the incubation time and on the amount of IAA-^{14}C injected. Hinman and Lang (1965), in a study of the products of the oxidation of IAA by horseradish peroxidase, found that the course of the reaction was highly dependent on the substrate concentration. The changeover came at concentrations in excess of 2×10^{-4} M. However, after allowing for dilution of the IAA-^{14}C solution in the endosperm, the concentrations employed here were all less than this. Furthermore, at all rates of application of IAA-^{14}C, there was a close similarity between the proportions of radioactivity exported into the shoot and roots, the distribution of the radioactivity within

the shoot, as well as the proportion of IAA metabolised in the grain. Therefore it seems likely that the proportion of different metabolites within the grain was also similar in each case.

Conjugation of exogenously applied IAA to form IAAsp is well known in plant tissues (reviews; Schneider and Wightman, 1974; Purves, 1978). However there is an increasing number of reports of IAA-^{14}C incorporation into complexes from which IAA-^{14}C can be released by mild alkaline hydrolysis e.g. in etiolated Zea shoots (Hall and Bandurski, 1978), pea stem segments (Davies, 1976), Nicotiana petiole slices (Liu, Gruenert and Knight, 1978) and leaf tissues of Citrus sinensis and Eucalyptus camaldulensis (Riov, Goren and Dror, 1981). More specifically, esterification of IAA-^{14}C with glucose has been shown in Colchicum leaves (Zenk, 1961) and in pea and bean stem segments (Davies, 1972). Kopcewicz et al. (1974) using maize and radio-GLC analysis, found labelled esters of myo-inositol, glucose and glucans were formed after incubation of mature kernels in dilute solutions of IAA-^{14}C. Compared to the amount of conjugation observed in this study, the proportion of IAA-^{14}C esterified in Kopcewicz and co-workers' investigation was very low (0.3%); most of the IAA-^{14}C remained free in the grain even after 24 h, and a small amount was metabolised to what were presumed to be oxidation products (Kopcewicz et al., 1974). However the maize grains were just beginning to germinate, whereas Avena grains which had been incubated for 63 h after sowing, were used here. Not only might the metabolic rates in the two experiments be expected to be different, but also very little dissolution of the endosperm would have occurred in the maize kernels. As is discussed below, most of the IAA metabolism appears to occur in the milky endosperm. The relationship of the IAA oxidation products found by Kopcewicz et al. (of which there were 3 main groups) and 'X' and 'Y' is unknown.

The most comparable study to the present investigation is that of Epstein et al. (1980), who applied IAA-^{14}C to

the cut surface of the endosperm of 4 d old etiolated Zea seedlings. After 8 h, only 17% of the applied radioactivity could be extracted from the grain as free IAA-^{14}C, which is similar to the values of 13 - 21% obtained in this investigation after 6 h incubation. Allowance must be made when comparing these figures, for the fact that, in Zea only 2.6% of the applied radioactivity was transported out of the grain into the shoot and roots (Hall and Bandurski, 1978), whereas in Avena this figure was approximately 13% (after 6 h incubation).

Epstein et al. (1980) did not examine the metabolites of IAA in detail. They established only that 14% of the IAA-^{14}C was decarboxylated and that the remainder (approximately 67%) was 'lost' i.e. it was neither extractable into ether as free IAA nor decarboxylated. This 'lost' radioactivity is designated by a question mark in their Fig. 3 (Epstein et al., 1980 p 419). These workers did not test their extract for alkali-labile bound forms of IAA-^{14}C and claimed, without any supporting evidence, that the 'lost' IAA was converted to non-Ehmann reagent-reactive, oxidation products, like those formed in germinating maize kernels (Kopcewicz et al., 1974). They apparently dismissed the possibility that the IAA had become esterified, because of the low rate of esterification their group had observed previously in germinating grains (Kopcewicz et al., 1974). However, the rate of IAA oxidation in germinating grains is also very low (Kopcewicz et al., 1974). Their hypothesis allows that a large increase in the rate of oxidation occurred as seedlings aged, yet ironically, excludes the possibility that a similar increase in the rate of conjugation could have occurred. It is therefore possible that part of Epstein and co-workers' 'lost' IAA-^{14}C was esterified, while part was catabolised, and thus the situation is similar to that occurring in Avena.

No comparison of decarboxylation with Epstein et al.'s study is possible, since IAA labelled in the 2-position was used in this investigation. Part of the decarboxylation

observed by Epstein et al. (1980) may have been caused by traces of sodium hypochlorite (NaOCl) adhering to the grain after surface sterilisation, since Abdul-Baki (1974) has shown that amino and organic acids, including IAA, are strongly decarboxylated by NaOCl. Even thorough washing does not remove NaOCl completely after sterilisation and this was a problem in ^{14}C incorporation studies. In accordance with the procedure recommended by Abdul-Baki, residual NaOCl was removed from the grains used in this work, by soaking them in 0.01 M HCl for 10 min after sterilisation.

In this study, some 2-4% of the applied radioactivity was recovered from the KOH solution in the bottom of the incubation test tubes in which the injected plants were suspended. This may represent $^{14}CO_2$ evolved from loss of the methyl carbon, although it may simply be radioactive guttation fluid secreted from the coleoptile tip, which has run down the side of the test tube. IAA-^{14}C injected into the endosperm of Avena seedlings has previously been shown to appear in the guttation fluid (Sheldrake, (1973).

In all the experiments, radioactivity was transported rapidly out of the grain, into the shoot and roots, and in a preliminary experiment, was detectable at the tip of the shoot within 5 min of injection. Since the radioactivity had to diffuse through 2-3 mm of endosperm and travel 30 mm up the shoot, this represents a translocation rate of at least 360 $mm.h^{-1}$. This velocity suggests translocation in the vascular system (Little and Blackman, 1963; Eschrich, 1968; Brossard and Tepper, 1969; Bonnemain, 1971; Morris and Kadir, 1972). Acid fuschin dye applied to the endosperm was found to be transported in the cortical vascular bundle in the mesocotyl, and to accumulate at the coleoptile tip. Similarly, dye introduced by the roots accumulated at the tip, but translocation in this instance was via the central vascular stele in the mesocotyl. In both cases, the accumulation of dye could be hastened by increasing the rate of transpiration using a fan, suggesting that translocation was occurring in the xylem. Evidence for the acropetal translocation of IAA and other substances in the xylem, from

endosperm to shoot in Avena, has previously been presented (Sheldrake and Northcote, 1968; Sheldrake 1973).

On the other hand, Whitehouse and Zalik (1967), using etiolated bean plants, concluded that transport of IAA-^{14}C in the epicotyl following injection into the colyledons, probably does not occur in the xylem. Transport of radioactivity could be inhibited by killing a ring of tissue in the epicotyl with heat treatment, but translocation of dye from the roots was unaffected, indicating that the xylem still remained intact. However, the rate of IAA-^{14}C transport observed in the normal bean plant was low, suggesting that translocation was probably by a different system to Avena.

Radioactivity was found to accumulate at the tip of the coleoptile following injection of IAA-^{14}C into the endosperm. Sheldrake (1973) has shown previously that radioactivity accumulates at the coleoptile tip when IAA-^{14}C is applied to the roots or to the cut base of the coleoptile; and the results obtained here support his claim that a similar build-up occurs, following application to the endosperm. These results, together with Sheldrake's and the dye-labelling experiments, indicate that accumulation at the tip occurs irrespective of whether translocation in the mesocotyl is via the cortical vascular bundle (from the scutellum), or via the central stele (from the roots). That is, these two bundles appear to function similarly, with respect to acropetal transport.

The build-up of radioactivity at the tip tended to follow, rather than precede, accumulation elsewhere in the shoot, particularly the mesocotyl. This suggests that the coleoptile tip is probably not acting as a site for the accumulation and redistribution of IAA and related compounds transported from the grain, as was postulated by Sheldrake (1973). Nevertheless, the evidence is in accord with Sheldrake's belief that acropetally moving substances build up at the coleoptile tip, mainly because the vascular bundles terminate in this region (Sheldrake, 1973). However, there is no indication that these compounds are then transported basipetally to other parts of the shoot.

The distribution of radioactivity in the rest of the shoot, observed in this investigation, does not correlate with the endogenous levels of IAA. The mesocotyl contained higher radiactivity levels than the coleoptile, whereas the distribution of endogenous IAA in the Avena shoot is almost the opposite of this; the coleoptile contains higher concentrations of IAA than the mesocotyl (Thimann, 1934; van Overbeek, 1938; Mousdale, cited by Malloch and Osborne, 1976). The distribution of radioactivity can be correlated with the abundance of vascular tissue. The highest levels of radioactivity occurred at the coleoptilar node and in the primary leaves, both of which contain numerous vascular bundles (Avery, 1930). Similarly, the higher concentration in the mesocotyl than the coleoptile can be accounted for by the fact that the Avena mesocotyl contains two major vascular bundles, while the coleoptile contains two minor bundles (Avery 1930; McCall, 1934).

Because the same proportion of the applied radioactivity was translocated, irrespective of the concentration of the IAA-^{14}C solution applied, it suggests a fixed proportion of the injected solution was transported into the shoot and roots in each case. Since none of the applications resulted in an increase in extension growth of either the shoot or roots (section 3.5.1.1), it is unlikely that IAA-^{14}C was being transported to fulfil specific growth requirements. It seems more likely that the radioactivity was carried along by the nutrient flow out of the endosperm, and that the amount transported merely reflects the proportion of radioactivity comprising the milky (liquid) endosperm at any particular time. This view is supported by the data of Hall and Bandurski (1978) which, after correction for counting efficiency, show that in 8 h, similar proportions of the radioactivity were transported into the shoot when either 3670 Bq of tryptophan-^{3}H or 35 Bq IAA-^{14}C was applied to the endosperm of Zea seedlings.

It is unlikely that the IAA-^{14}C conjugate was transported into the shoot, because in Zea, the IAA glucans, which are high molecular weight substances similar to those

described here (Percival and Bandurski, 1976), are non-transportable (Epstein et al., 1980). The results of the 2 kBq experiment indicate that initially free IAA was transported, but that the amount declined progressively wth time. After 6 h, only traces were apparent in the scutellum and, after 24 h, no IAA at all was detectable in the shoot, suggesting that translocation of IAA had ceased. The balance of the radioactivity translocated was probably metabolite 'Y' that was formed in the grain, and perhaps 'X', but since its identity is uncertain, it is unclear whether 'X' is readily transportable.

Once in the shoot, IAA was rapidly metabolised. Some metabolism may have occurred in the vascular system during translocation, but the presence of a considerable quantity of free IAA at the coleoptile tip in the early stages of the 2 kBq experiment, indicates much of the IAA-^{14}C was transported unaltered. Since the vascular bundles terminate 0.5 - 0.7 mm from the extreme tip of the coleoptile (Sheldrake, 1973) the remainder of the metabolism must have occurred in the tissues of the coleoptile tip. Presumably the metabolism in the mesocotyl also occurred in the tissues outside the vascular bundles.

It is unlikely that the observed metabolism of IAA is solely a detoxification response due to application of excess IAA, since, even at the lowest application rate, metabolism was extremely rapid. Although, it is possible that at the higher applications of IAA-^{14}C, the rate of metabolism was stimulated to some extent. Using spectrofluorometric means, Mousdale (cited by Malloch and Osborne, 1976) has calculated the concentration of endogenous IAA to be 20 ng.g^{-1} fresh weight in the coleoptile, and 10 ng.g^{-1} in the mesocotyl, of 3 d old etiolated oat seedlings. This concentration is assumed to remain approximately constant as the shoot develops (Hall and Bandurski, 1978). Since at age 3 d, the coleoptile and the mesocotyl each account for approximately half of the fresh weight of the shoot, Mousdale's figures agree closely with the overall IAA concentration of 16 ng.g^{-1} f.wt, estimated by Bandurski and Schulze (1977) for the Avena shoot.

Six hours after injection, the mesocotyl (excluding the scutellum) weighed 22 mg (Table 3.16). At a concentration of 10 $ng.g^{-1}$, this represents 0.22 ng IAA.$mesocotyl^{-1}$. The coleoptile at the same stage weighed 16 mg, and at a concentration of 20 $ng.g^{-1}$, would contain 0.32 ng IAA. In the 6 h period after injection of 20 Bq IAA-^{14}C into the endosperm, 1.14 Bq and 0.6 Bq of radioactivity moved into the mesocotyl and the coleoptile, respectively. If all of the radioactivity was in the form of IAA (at an IAA-^{14}C specific activity of 11.28 $Bq.ng^{-1}$), this is equivalent to 0.10 ng IAA.$mesocotyl^{-1}$ and 0.05 ng IAA.$coleoptile^{-1}$. This represents approximately a 45% perturbation of the endogenous IAA levels in the mesocotyl, and a 16% perturbation in the coleoptile. Yet in both tissues the applied IAA was totally metabolised after 6 h. The degrees of perturbation calculated here are undoubtedly over-estimates, because not all of the radioactivity transported was IAA-^{14}C, and the influx into the shoot was spread over a 6 h period. Also, according to the figures of Zimmermann and Rudiger (1976), the endogenous IAA concentration in the Avena shoot is about 6 times higher than estimated by Mousdale, (cited by Malloch and Osborne, 1976) and Bandurski and Schulze (1977). It thus seems unlikely that, at the lowest application rate at least, IAA-^{14}C is metabolised in response to overloading of the endogenous IAA levels.

Similarly, the disappearance of IAA in the shoot is not related to the particular method of application employed in this study. Rapid metabolism also occurs when IAA-^{14}C is applied to the tip of intact Avena coleoptiles (Shen-Miller, Cooper and Gordon, 1969) and to the apical cut surface of coleoptile and mesocotyl segments (Malloch and Obsorne, 1976).

Exogenously-applied IAA can obviously enter the cells in the shoot, as evidenced by the growth responses obtained in the standard Avena curvature and straight growth tests (e.g. Thimann, 1969), and when IAA is applied to the coleoptile tip of intact oat seedlings (Went, 1935; and in this study) and to the grain (this study). As labelled and

unlabelled IAA are assumed to be biologically indistingishable, the results obtained here would indicate that the endogenous IAA in the shoot is also rapidly metabolised, in which case all of the IAA in the coleoptile and mesocotyl is probably lost over a 6 h period. This represents metabolism of about 0.036 ng IAA.h^{-1} in the mesocotyl and 0.053 ng IAA.h^{-1} in the coleoptile. Although, as noted above, at the higher applications of IAA-^{14}C this rate of metabolism is probably accelerated. These estimated rates of metabolism are well within the bounds of the rate of metabolism of endogenous IAA (0.4 ng.h^{-1}), observed in coleoptiles of 3 d old etiolated Zea seedlings (Iino and Carr, 1982a). Destruction of auxin in intact coleoptiles was also reported by earlier workers, where it was described as auxin 'used in growth' (Bonner and Thimann, 1935; Went, 1935; van Overbeek, 1938).

The radioactive metabolites in the shoot were not characterised in detail. The majority of the radioactivity was extracted by 95% ethanol, although a small amount was soluble in water and NaOH. The ethanol-soluble radioactivity was immobile when chromatographed in isopropanol/ammonia/water (10:1:1 v/v/v) and methyl ethyl ketone/hexane (35:65 v/v) - a property common to all of the IAA metabolites formed in the grain. This immobility excludes the metabolites from being products such as indole-3-acetaldehyde or indole-3-aldehyde, which are very mobile in these solvent systems (Fletcher and Zalik, 1965; Morris et al, 1969; Stahl, 1969).

The metabolites in the shoot came from two sources; those formed in the grain and transported into the shoot (probably 'X' and 'Y'), and those derived from the metabolism of IAA in the shoot itself. It is unlikely that much of the turnover of IAA in the shoot consists of conjugation. Two endogenous bound forms of IAA have been identified in etiolated Avena shoots: esterified IAA, accounting for 5.5% of the total IAA, and peptidyl-bound IAA, comprising 76.5% (Bandurski and Schulze, 1977). At the rates of IAA metabolism discussed above, if IAA was being conjugated then, over time, a substantial increase in the levels of endogenous

bound IAA would be expected. However, this does not occur (Nowacki and Bandurski, 1980; Iino and Carr, 1982a); although, it is likely that a small amount of conjugation occurs to maintain the IAA-ester and IAA-peptide concentrations as the shoot grows. A small amount of esterification has been reported in etiolated Zea shoots following application of IAA-^{14}C to the endosperm (Hall and Bandurski, 1978). According to Iino and Carr (1982a), decomposition is probably the major process by which IAA is lost, in the Zea coleoptile at least. Recently, using HPLC and GC purification and identification techniques, Reinecke and Bandurski (1981) have shown that, following application to the grain of Zea, the major metabolite of IAA-^{14}C in the shoot is oxindole-3-acetic acid. Whether this product is also formed in Avena is unknown.

Although the metabolism of IAA-^{14}C in the mesocotyl and coleoptile probably reflects the turnover of endogenous IAA in these tissues, the observed transport of IAA-^{14}C into the shoot, and much of the metabolism of IAA-^{14}C in the grain, almost certainly is not representative of the normal situation in the Avena seedling. In all experiments, translocation of radioactivity out of the grain into the shoot and roots was rapid in the first hour, but then declined to a much slower rate, even though the growth rate of the shoot (and presumably the roots) remained constant. From the 2 kBq experiment it is apparent that a similar pattern of decline in the rate of IAA metabolism also occurred. Even though within an hour of injection, over 50% of the applied IAA was metabolised, the rate slowed considerably thereafter; and by the end of the 24 h experimental period, 12% of the original IAA still remained free in the grain (in addition IAA-^{14}C was being exported into shoot and roots).

The declines in the rates of transport and metabolism were not simply due to lack of IAA in the grain, because in all experiments, after 6 h the amount of free IAA had only fallen to about 1/6 th of the original, yet the rates of

transport and metabolism had decreased by more than 20-fold. Nor was the decrease due to saturation of the transport and metabolic capabilities of the seedling, as similar patterns were evident at all IAA application rates. It appears that some of the applied IAA was becoming unavailable for transport and was also immune from metabolic attack. 'Auxin protectors' (Stonier, 1972) cannot account for the reduction in metabolism, since these compounds merely delay IAA oxidation for 1 to 2 h. The evidence suggests that the IAA was becoming compartmentalised in some way, and was both unavailable for transport, and protected from conjugation and degradation.

The most likely explanation is that after injection, part of the applied IAA-^{14}C finds its way into the intact cells of the endosperm. In cereal grains, dissolution of the cell walls in the endosperm occurs first near the scutellum, and gradually extends to the remainder of the endosperm (Pollock, 1962; Epstein et al., 1980). Under malting conditions complete degradation in barley may take 10 days (Brown and Morris, 1890). At the stage used in this investigation (63 h), the endosperm of the oat seedlings was still quite firm, suggesting that cell wall disintegration was only partial. The above explanation would account for the fact that, 6 h after injection, similar proportions of IAA remained unmetabolised at each application rate; and that the export of radioactivity from the grain decreased after a period, in all cases. The slow but continued metabolism of IAA-^{14}C, and transport of labelled material into the shoot and roots in the last 20 h of the experiments, is interpreted as the gradual release of IAA (and its readily mobile metabolites) from the endosperm as cell wall degradation proceeded.

The initial rapid transport of radioactivity is an indication that much of the injected solution went into the liquid part of the endosperm adjacent to the scutellum. And the initial rapid rate of metabolism suggests that IAA in the free form does not normally exist in the milky endosperm. That IAA is metabolised in the milky endosperm is

supported by data from Bandurski's group. Very little IAA-^{14}C metabolism occurs in Zea grains which are commencing germination (Kopcewicz et al., 1974), when the endosperm is mostly solid, but IAA-^{14}C is rapidly metabolised in grains of 4 d old seedlings, where liquefaction of the endosperm is well advanced (Epstein et al., 1980).

As in the shoot, it is unlikely that the rapid metabolism of IAA in the grain was due to application of excess amounts of growth regulator. The 2 kBq, 200 Bq and 20 Bq injections of IAA-^{14}C contained 177, 17.7 and 1.77 ng of IAA respectively. Obtaining an accurate estimate of the amount of endogenous IAA in Avena grains, and therefore the degree of perturbation that injection involved, is difficult. Percival and Bandurski (1976) have estimated that dehusked grains of cv. 'Lodi' oats contain 440 $\mu g.kg^{-1}$ free IAA, but they give no indication of how much this represents per grain. One kg of (naked) grains of the cv. 'Terra' oats used in this study contains approximately 30,000 grains. Using Percival and Bandurski's figures, this represents about 15 $ng.grain^{-1}$ of free IAA. According to Zimmermann et al. (1976), the free IAA content of cv. Sorte Delphin grains, with hulls intact, is about 1150 $\mu g.kg^{-1}$. Since 1 kg of cv. 'Mapua' grains (with husks) contains approximately 23,000 grains, an approximate estimate of the amount of free IAA per grain, on this basis, would be 50 ng.

Both Zimmermann et al. (1976) and Percival and Bandurski (1976) found that in mature Avena grains, bound IAA levels (i.e. 1 N NaOH-hydrolysable) were 15-16 times higher than those of free IAA. During the first 4 d of seedling growth, IAA levels in the grain remain essentially constant, but the amount of bound IAA declines steadily (Zimmermann et al., 1976).

Assuming a free IAA content of between 15-50 ng. $grain^{-1}$, injection of 177, 17.7 and 1.77 ng IAA into the grain would have increased the endogenous IAA levels by 350 - 1180%, 35 - 118% and 3.5 - 11.8%, respectively. At the lowest application rate, even if only part of the

endogenous IAA were in the liquid part of the endosperm, there would have been little disturbance from the IAA-^{14}C injected, yet the proportion metabolised was similar to when higher amounts of IAA were applied. Furthermore, Epstein et al. (1980) found a similar level of IAA-^{14}C metabolism in Zea grains, even though the expected perturbation of the endogenous pool from their application was only 3%.

It is possible that the metabolism of IAA was in response to tissue damage during injection. However, as the needle wound was very small and the radioactivity quickly moved away from the injection area (indicated by its rapid appearance in the shoot), it is unlikely that this would account for the large amount of metabolism observed. Another possibility is that forcing solution into the endosperm may have increased the flow of nutrients out of the endosperm, by creating pressure within the grain. The grain was found to contain 43 µl of water, most of it presumably in the endosperm. If half the endosperm was liquefied, injection of 4 µl of IAA-^{14}C solution could have increased the volume in the fluid part of the grain by about 20%. It is doubtful whether such an increase would be sufficient to account for the extremely high initial rate of transport out of the grain.

In summary, it seems that upon injection, much of the IAA-^{14}C diffuses into the milky endosperm, where it is rapidly metabolised, and from where IAA and its transportable derivatives are carried into the shoot and roots. The decline in both the rate of metabolism of IAA and the transport of radioactivity, is consistent with IAA and its low molecular weight metabolites becoming incorporated into some of the intact cells in the endosperm.

The fact that even small quantities of IAA are metabolised in the liquid part of the endosperm suggests that IAA does not normally occur in the free form in this part of the grain. Therefore, both the initial rate of metabolism, and of IAA transport, are almost certainly artefacts, and not true indications of the behaviour of endogenous IAA in the grain. At the lowest injection rate,

which represents amounts of IAA that are physiologically realistic, about half of the free IAA injected disappeared from the grain in one hour. This represents a loss of about 0.9 ng $IAA.h^{-1}$. If the labelled IAA were mixing with the 17-50 ng of endogenous IAA calculated to be present in the grain, then the total amount of IAA metabolised in an hour would be in the order of 8.6 - 25 ng. Since this would exhaust the supply of free IAA in the grain in 0.7 - 5.8 h, this initial response to injection of IAA-^{14}C clearly is not physiologically significant.

If free IAA exists in the endosperm, then it must do so in the intact cells. Therefore the slower rate of IAA metabolism and transport of radioactivity in the 21-24 h period after injection, presumably as the cells are lysed, is probably more representative of endogenous IAA transport and metabolism. In all the labelling experiments, IAA-^{14}C was absent or virtually absent from the shoot 6 h after injection, even though IAA still comprised about 20% of the radioactivity in the grain. Since free IAA was disappearing from the grain, it was obviously being metabolised *en route*. Most of this metabolism probably occurred in the milky endosperm, because IAA-^{14}C liberated from the intact endosperm cells, had to migrate through the liquid portion prior to reaching the shoot. Furthermore, 6 h after injection of 2 kBq IAA-^{14}C, only 2% of the radioactivity in the scutellum was found to be as free IAA. On the basis of the above arguments, it is concluded that transport of endogenous free IAA, from the endosperm into the shoot and roots, does not occur to any significant extent. A similar conclusion was reached by Hall and Bandurski (1978) following experiments in which radioactively-labelled IAA was applied to the cut endosperm surface of 4 d old *Zea* seedlings. They also found that only traces of IAA-^{14}C could be detected in the radioactivity extractable from the shoot 8 h after IAA application.

Sheldrake (1973) has presented convincing evidence that in *Avena* substantial quantities of IAA (and other auxins) are transported acropetally in the shoot, probably

from the grain. He estimated, by bioassay, that about 0.004 ng of auxin (as IAA equivalents) appears in the guttation fluid of each coleoptile, each hour, and that about half of this is IAA. By collecting fluid from coleoptiles which had been repeatedly decapitated, he showed that this auxin is actually a constituent of the xylem sap itself and not merely eluted from the coleoptile tip. As IAA-^{14}C injected into the endosperm appeared at the coleoptile tip and in the guttation fluid, Sheldrake considered the endosperm was the source of the free IAA. The results of the present investigation however indicate this is unlikely.

One possible source of Sheldrake's acropetally-moving endogenous IAA is the aleurone layer and/or pericarp. Most authors have implicitly assumed that IAA is localised in the starchy endosperm in cereal grains (Sheldrake, 1973; Hall and Bandurski, 1978; Nowacki and Bandurski, 1980; Epstein et al., 1980). However, Hatcher (1945) found 71% of the total auxin activity (free + bound) extracted from rye grains was localised in the aleurone layer. Similarly, rice polishings (i.e. aleurone and pericarp) have been shown to contain considerable auxin (Went and Thimann, 1937); and polished barley contains much lower amounts of IAA (both free and bound) than other cereal grains, which have not been polished (Bandurski and Schulze, 1977).

Because of the rapid metabolism of IAA that has been shown to occur in the starchy endosperm, the pool of free IAA in the grain may be compartmentalised in tissues such as the aleurone layer and pericarp, and thereby be physically separated from the enzymes responsible for destruction and conjugation. In barley, the cells of the aleurone layer have been shown to retain their integrity, even when hydrolysis of the endosperm is complete (Pollock, 1962). It is possible, therefore, that IAA contained within the aleurone layer (and pericarp) is transported within these cells, into the scutellum and then into the shoot. However, as the amount of free IAA in the Avena grain remains essentially constant (Zimmermann et al, 1976), it is unlikely that such IAA is transported into the shoot in significant quantities.

Nevertheless, localisation of free IAA in the aleurone layer and pericarp may account for Epstein and co-workers finding that the pool of free IAA in the Zea endosperm appears to be continuously replaced over a 3-4 h cycle (Epstein et al., 1980). Using an isotope dilution technique, they determined that following application of IAA-^{3}H to the endosperm, the ratio of labelled: non-labelled IAA rapidly declined, although the total amount of IAA remained approximately constant. This they interpreted as evidence that the pool of free IAA in the endosperm was constantly turning over. However, they could offer no explanation as to why this was occurring. The apparent turnover of IAA observed by Epstein et al. may be an artefact. If the endogenous pool of IAA was contained in the aleurone layer and/or pericarp, then labelled IAA applied to the endosperm could be perceived as 'foreign' and be rapidly metabolised. However, the endogenous pool might remain unaltered. Thus, upon extraction, the decline in the proportion of labelled: unlabelled IAA would give the impression of turnover of endogenous IAA.

The most likely source of the free IAA detected by Sheldrake (1973) in Avena guttation fluid, is from hydrolysis of the non-transportable IAA-glucoprotein ester in the endosperm. Such a function for the IAA-conjugate in Avena grains has previously been suggested by Zimmermann et al. (1976). The conjugate itself is probably not translocated due to its high molecular weight (Percival and Bandurski, 1976; Epstein et al., 1980). However, hydrolytic release of IAA at the scutellar surface could account for the observed transport of free IAA in the shoot. The amount of conjugated IAA in the Avena grains has been shown to fall gradually and this decline is accompanied by a corresponding rise in the IAA content of the shoot (Zimmermann et al., 1976).

In contrast, Bandurski and co-workers claim that, in Zea, intact IAA-ester is transported into the shoot. These researchers have developed an hypothesis, based on the

results of tracer experiments using IAA-^{14}C-*myo*-inositol, that this low molecular weight ester (a major component of the IAA-conjugates in the *Zea* endosperm) is transported into the shoot and there serves as the source of free IAA (Epstein *et al*, 1980; Nowacki and Bandurski, 1980). The apparent production of IAA by coleoptile tips, they explain in terms of hydrolysis of accumulated IAA-*myo*-inositol. As in this investigation, their IAA-labelling experiments led them to conclude that the transport of free IAA directly from the endosperm into the shoot, is of minor significance (Hall and Bandurski, 1978).

There is, however, a number of difficulties with the IAA ester transport theory as proposed by Bandurski's group. Their estimate of the amount of IAA supplied by transport of *myo*-inositol from the grain (1.11 ng.h^{-1}, Nowacki and Bandurski, 1980) is considerably less than their previously published estimate of the IAA requirement of the shoot (1.62 ng.h^{-1}, Hall and Bandurski, 1978). They later claimed that this estimate of IAA requirement was too high (Nowacki and Bandurski, 1980), but gave no indication of why. Furthermore, Hall and Bandurski's estimate is only of the amount of IAA needed to maintain a constant concentration as the shoot increases in size, and assumes there is no breakdown of IAA. However, considerable destruction of IAA occurs in the *Zea* coleoptile at least (Iino and Carr, 1982a), and so the IAA requirement of the shoot must be much higher than hypothesised by Bandurski and co-workers.

Another difficulty with Bandurski's hypothesis is that the amount of IAA that can be obtained from *Zea* coleoptile tips by diffusion, far exceeds the conjugated IAA content of the entire coleoptile, including the primary leaves (Iino and Carr, 1982a). Using modern spectrofluorometric techniques, Iino and Carr have recently repeated much of the 'classical' auxin work of Went, van Overbeek and others. While not disputing that IAA-ester may be transported from the grain to the shoot, they claim that it is not the source of free IAA in the shoot (Iino and Carr, 1982b).

Iino and Carr (1982b) also found that the free IAA content of isolated coleoptile segments fell then increased again without any change occurring in the amount of conjugate. While this experiment is not definitive, Bandurski and co-workers have little evidence to support their assertion that IAA-myo-inositol actually gives rise to free IAA in the shoot. The small amount of IAA-^{14}C released from labelled IAA-myo-inositol in vivo (Nowacki and Bandurski, 1980) could simply have been the result of inadvertent hydrolysis of the ester during extraction and purification (Ueda and Bandurski, 1969; Bandurski and Schulze, 1974; Nowacki and Bandurski, 1980).

Also, as a general hypothesis for cereal seedlings, the transport of esterified IAA into the shoot, where it releases free IAA, is not tenable. In Avena, almost 90% of the IAA-ester in the grain is in the form of non-transportable, high molecular weight, IAA-glucoproteins (Percival and Bandurski, 1976; Epstein et al., 1980). Sheldrake (1973) found IAA esters were almost completely lacking from the guttation fluid collected from Avena coleoptiles. Furthermore, the bulk of the conjugated IAA in the shoot consists of peptide-bound IAA, which is not detectable in the grain; IAA-esters comprise only about 5% of the IAA in the shoot (Bandurski and Schulze, 1977).

Moreover, Bandurski's hypothesis does not take account of the large quantities of IAA present in the vascular system of Zea seedlings. Sheldrake (1973) found that although IAA-ester occurred in Zea guttation fluid, free IAA was more prevalent. Also, some of Bandurski's own data show that the vascular stele of etiolated Zea mesocotyls contain particularly high concentrations of free IAA, but lesser amounts of IAA-ester (Bandurski, Schulze and Hall, 1980; Pengelly and Bandurski, 1981).

It is possible that in Zea, as postulated for Avena, hydrolysis of the non-transportable IAA-esters occurs at the scutellum, and that these are the source of the free IAA in the shoot. Epstein et al. (1980) found 135 pmol of IAA-glucan disappeared from the endosperm of 4 d old Zea

seedlings every hour, Hydrolysis of this ester would be more than sufficient to supply the 9 pmol (or more) of IAA required by the Zea shoot each hour (Epstein et al, 1980).

The very fact that large quantities of IAA-esters exist in cereal grains has led to speculation that they serve some function in development of the seedling (Went and Thimann, 1937; Sheldrake, 1973; Bandurski, 1980; Trewavas, 1981). Furthermore, these esters increase in amount during maturation of the grain (Hatcher, 1943; Bandurski, quoted in Epstein et al., 1980), and decline during germination (Ueda and Bandurski, 1969; Zimmermann, et al., 1976; Epstein et al., 1980). As has been noted by Trewavas (1981), the etiolated seedling obtains all of its organic materials from the endosperm, and it is therefore likely that it obtains at least part of its IAA also in this manner.

Iino and Carr (1982, a and b), however, believe the grain does not contribute appreciable quantities of free IAA to the shoot of Zea, either as free IAA or from IAA-esters. Their studies support the so-called classical auxin hypothesis (e.g. Wildman and Bonner, 1948): that the coleoptile tip (and to a lesser extent the primary leaves) is the source of free IAA, which is synthesised from either tryptophan or tryptamine. They found the amount of IAA obtained from the coleoptile tip by diffusion, far exceeded the IAA-ester content of the entire coleoptile, and that sufficient IAA diffused from the base of the coleoptile to supply the needs of the mesocotyl. In support of the latter, they showed that decapitation of the coleoptile resulted in inhibition of mesocotyl extension, a reduction in the amount of IAA diffusing out of the base of the coleoptile, and a decrease in the concentration of free IAA in the apical portion of the mesocotyl.

Iino and Carr discounted the possibility that the grain or roots supplied significant amounts of free IAA to the mesocotyl. When whole shoots were excised, and thereby deprived of an acropetal supply of IAA, the free IAA content in the mesocotyl remained unchanged over a 4 h period, suggesting that the concentration of IAA was maintained by diffusion from the coleoptile (Iino and Carr, 1982b).

However, this experiment is not conclusive, as seedlings from which the grains have been removed, quickly cease growing (Jackson, unpublished). Also, the rate of IAA metabolism in excised Zea mesocotyl segments has been shown to be quite low (Malloch and Osborne, 1976). Thus, the amount of IAA 'used' in the excised mesocotyl in 4 h may be small and easily replaced by diffusion from the coleoptile. Iino and Carr's experiment, therefore, does not exclude the possibility that in the intact seedling, some IAA is supplied from the grain to the mesocotyl, and to the coleoptile.

There is also a number of difficulties with the classical hypothesis, some of which have been raised previously (Sheldrake, 1973; Trewavas, 1981). Perhaps the most controversial point is whether sterile coleoptile tips are capable of synthesising substantial amounts of IAA from tryptophan, or whether the apparent production is due to contaminating micro-organisms (see Trewavas, 1981). Sheldrake (1973) has found that tryptophan is absent from Avena guttation fluid, suggesting it is not transported from the endosperm. Also, when Hall and Bandurski (1978) applied labelled tryptophan to the endosperm of etiolated Zea seedlings, relatively little labelled IAA appeared in the shoot.

There are thus two distinctly different schools of thought with regard to the origin of free IAA in the shoot of cereal seedlings. One school (Sheldrake, Bandurski, Trewavas) beleives that IAA and/or IAA-ester in the grain is the source of IAA in the shoot, and discount synthesis at the coleoptile tip. The other, the 'classical' school, supported by Iino and Carr, believesthe shoot produces all of its IAA requirements and is independent of a supply of IAA or IAA-ester from the grain. These two hypotheses are diametrically opposed, yet both groups have presented some good evidence in support of their particular view.

The most likely explanation is that neither dogma is correct on its own, and that there are two sources of the IAA in the cereal shoot - the coleoptile tip and the grain. This simple hypothesis would account for many of the apparent anomalies that arise if either of the other two hypotheses is adopted alone. The relative importance of

the two sources of IAA may differ between species of the Gramineae. In Zea, Iino and Carr (1982, a and b) have produced convincing evidence that the apical part of the mesocotyl is supplied with IAA from the coleoptile. Removal of the coleoptile tip reduces the amount of IAA diffusing from the coleoptile into the mesocotyl, and decreases the concentration of IAA in the tissues of the upper mesocotyl. Presumably the IAA moving from the grain in Zea (Sheldrake, 1973), supplies the rest of the mesocotyl and perhaps, to some extent, also the apical part of the mesocotyl. The role of conjugated IAA, transported from the endosperm, is unclear.

The auxin economy of Avena shoots appears to be markedly different from that of Zea. There is probably little or no direct translocation of IAA-ester from the endosperm to the shoot in Avena. Also, the mesocotyl appears to be independent of a supply of IAA from the coleoptile, since even repeated decapitation of the coleoptile does not affect mesocotyl extension (Mer, 1951). Furthermore, relative to the endogenous IAA content of the respective shoots (Bandurski and Schulze, 1977), acropetal transport of auxin from the grain is much higher in Avena than in Zea, and, as noted above, the bulk of this auxin in Avena is free IAA (Sheldrake, 1973).

It is unlikely that IAA from the endosperm accounts for all of the free IAA in the Avena shoot. IAA from the tip appears to be important in extension growth of the Avena coleoptile (e.g. Boysen Jensen, 1936; Went and Thimann, 1937). Also, if it is assumed the distribution of radio-activity in the shoot observed here, reflects the accumulation of any compound moving acropetally from the endosperm, then transport from the grain alone could not account for the distribution of endogenous IAA in the Avena shoot (e.g. Mousdale, quoted by Malloch and Osborne, 1976).

What has been attempted here is to develop a reasonable working hypothesis to explain many of the apparently contra-dictory findings in the field of auxin physiology in cereal seedlings. I believe the 'dual-source' hypothesis for IAA

can explain many of these contradictions. Undoubtedly, there are findings which may not fit this hypothesis. Nevertheless, such exceptions are probably minor compared to the large body of evidence that must be set aside in each case, in order to accept either the 'Seed Precursor' or the 'Classical' hypothesis on its own.

Clearly, care needs to be taken when extrapolating the conclusions of studies on one species of Gramineae to another. Much of the confusion surrounding the alleged role of the coleoptile tip in controlling mesocotyl elongation in Avena has probably arisen because workers have assumed the regulatory systems in Zea and Avena are similar. However, anatomically and biochemically the 2 species are quite distinct - for example, photosynthesis in Zea is based on a 4 carbon cycle, whereas in Avena the 3-C Calvin-Benson cycle is involved; the two species differ considerably in terms of anatomy, particularly with respect to the mesocotyl; and the nature of the IAA-conjugates in both the grain and shoot is clearly different.

Most of the 'classical' work on control of coleoptile elongation was performed using Avena seedlings (see Went and Thimann, 1937). However, when the 'classical' hypothesis was extended to include the regulation of mesocotyl extension (van Overbeek, 1935, 1936), most of the confirmatory experiments were done with Zea (e.g. van Overbeek, 1935, 1936, 1938; Inge and Loomis, 1937). This was presumably because the mesocotyl of these plants was more robust. Comments indicating this may be true were made by van Overbeek (1936).

The 'classical' evidence which implicated the coleoptile tip in the control of mesocotyl growth, as stated in the 'Introduction', was based on the observations that treatments which resulted in inhibition of mesocotyl elongation (decapitation, exposure to light, heat treatment), also resulted in a decrease in auxin supply, and inhibition could be totally or partially countered by applying exogenous IAA

to the coleoptile tip or stump. However, only in one study, using Zea, was it shown that the treatment which inhibited mesocotyl elongation (in this case, decapitation), resulted in a decrease in the concentration of auxin in the mesocotyl (van Overbeek, 1938). The evidence for the effect of illumination and heat treatment was more circumstantial; either simply that the inhibition could be reversed by application of exogenous IAA (Inge and Loomis, 1937) or, in addition to this, that auxin production from the coleoptile tip was shown to be less in treated plants (van Overbeek, 1936).

Since heat treatment, illumination and decapitation were all found to inhibit mesocotyl elongation in oats as well (see Mer, 1951), the auxin hypothesis was extended to include Avena (e.g. Went and Thimann, 1937). However, subsequent studies on the effect of brief light exposure, indicated that photo-inhibition of mesocotyl elongation in Avena could not be explained in terms of such an auxin hypothesis (Goodwin, 1941; Schneider, 1941; Mer, 1969). A similar conclusion was also reached by Mer (1969) with respect to heat treatment of Avena seedlings. Moreover, according to Mer (1969), earlier workers who claimed that decapitation of the coleoptile inhibited mesocotyl elongation, almost certainly exposed their plants to dim red light to facilitate manipulation, and thus confounded the effects of decapitation with the effects of light on mesocotyl growth. Mer (1951) has clearly shown that if the use of light is avoided during decapitation, Avena mesocotyl extension is unaffected.

The decapitation effect in Zea appears to be 'real', however. Iino and Carr's recent experiments (Iino and Carr, 1982 a and b), which repeat some of the 'classical' work in Zea seedlings, used an infra red 'safelight' which they claim is physiologically 'safe' (Iino and Carr, 1981). They found that decapitation of the Zea coleoptile does inhibit extension of the mesocotyl; and as well, reduces the amount of IAA secreted from the base of the coleoptile, and lowers the concentration of IAA in the apical portion of the mesocotyl (Iino and Carr, 1982b).

There is an obvious need to also repeat and extend the early work on light- and heat-induced mesocotyl inhibition in Zea, using less equivocal methods of IAA analysis - preferably in conjunction with similar, comparative studies in Avena. Furthermore, morphological studies like those already performed in Avena, are required in Zea - to determine whether the effects of light and heat treatment on cell elongation and division in the mesocotyl, are actually consistent with the 'classical' auxin hypothesis.

It is shown here that the transport of free IAA directly from the endosperm into the shoot in Avena, as proposed by Sheldrake (1973), is of minor significance. However, the hypothesis that the high molecular weight, non-transportable IAA esters in the grains of Zea and Avena are the source of free IAA in the shoot, is speculative and requires experimental verification. If further work confirms that the labelled IAA-conjugate formed in the grain of Avena is in fact IAA-glucoprotein, then the re-application of this labelled ester to the endosperm would answer this question.

However, before further feeding experiments are undertaken, it is imperative that the precise locations of the IAA-containing substances in the grain are determined. To this end, analytical techniques such as radioimmunoassay (e.g. Pengelly, Bandurski and Schulze, 1980; Pengelly and Bandurski, 1981), which require only small amounts of sample, would be ideal.

Where application of labelled substances to the endosperm is required, the use of plants in which the endosperm is totally liquefied would be desirable, since this would reduce the problem of compartmentalisation within the endosperm. Similar comments have been made by Epstein et al. (1980). Unfortunately, in Avena, at least, at the stage at which the endosperm is totally milky, mesocotyl extension has probably ceased. This may significantly alter the composition of the substances translocated out of the grain. As a compromise, younger plants could be used and the label applied to the end of the grain distal to the

scutellum. Since the labelled compound would have to diffuse through the solid part of the endosperm, *en route* to the scutellum, the release of radiactivity into the milky endosperm would probably be more controlled, and better mixing with the contents of the endosperm would result.

5.0 GENERAL CONCLUSIONS

(1) Rapid destruction of IAA occurs on silica gel TLC plates. This process is accelerated by light.

(2) Breakdown is so rapid (over 25% in 15 min) that during the normal course of sample application to the chromatogram, considerable 'losses' of IAA may occur. In IAA-^{14}C metabolism studies, the TLC breakdown products may be confounded with metabolites of IAA formed in the plant. Unless an internal standard is used, losses during application of IAA solutions to TLC plates may lead to serious underestimation of the endogenous IAA content of plant extracts. Potentially more serious in endogenous studies, is destruction of IAA on the plate after development, but prior to elution of the IAA spot for quantification, since this 'loss' may not be indicated by a labelled internal standard.

(3) Brief exposure to red light inhibits Avena mesocotyl extension by reducing cell division and the extension of certain cells in the apical region of the mesocotyl. This inhibition can be partially reversed by application of IAA to the coleoptile tip or to the endosperm. GA_3 application to the endosperm also partly counteracts red light inhibition.

(4) Cells in the mesocotyl are affected differently by red light. A reduction in the supply of IAA (or other growth substances) to the mesocotyl could not account for this. Brief exposure to light may affect the sensitivity of certain cells to IAA (or other growth substances).

(5) IAA-^{14}C injected into the endosperm of Avena seedlings is rapidly metabolised. One derivative consists of a complex containing carbohydrate, peptide and possibly phenolic substances, from which IAA-^{14}C can be released by mild alkaline hydrolysis. This complex is similar to an endogenous IAA-ester which was isolated from untreated Avena grains. Both the labelled and endogenous complex have some properties in common with the IAA-glucoprotein in mature Avena grains, which was partially characterised by Percival and Bandurski,

(1976). The identity of the two other main IAA-^{14}C derivatives formed in the endosperm, is unclear.

(6) Metabolism of IAA-^{14}C applied to the grain occurs mainly in the milky endosperm. The endogenous pool of IAA may be compartmentalised to prevent metabolism, possibly in the aleurone layer and/or pericarp.

(7) Radioactivity accumulates at the coleoptile tip following injection of IAA-^{14}C into the endosperm. Accumulation follows rather than precedes the build-up of radioactivity in the mesocotyl. Therefore, the coleoptile tip probably does not act as the site of accumulation and redistribution of IAA and associated compounds, as was suggested by Sheldrake (1973). Dye travelling acropetally in either the cortical vascular bundle or the central stele in the mesocotyl, also accumulates at the coleoptile tip.

(8) Translocation of free IAA directly from the endosperm to the shoot in Avena is of minor significance. The source of acropetally-moving IAA in cereal seedlings may be hydrolysis of the non-transportable IAA-esters at the endosperm-scutellar interface.

(9) IAA derived from the grain, and IAA produced in the coleoptile tip, both appear to be sources of IAA in Zea and Avena shoots. The relative importance of each source may differ between species, with production at the coleoptile tip being more important in Zea than in Avena .

(10) Anatomical and physiological differences between Avena and Zea seedlings mean that results of studies on one or other species, are not necessarily interchangeable. Failure to recognise this may have retarded the understanding of auxin physiology in cereal seedlings, particularly with regard to the importance of the coleoptile tip in controlling mesocotyl extension.

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